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PSYCHONEUROENDOCRINOLOGY OF SPAWNING BEHAVIOUR IN THE MALE GOLDFISH

by

ANN L. KYLE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **PSYCHONEURO-ENDOCRINOLOGY OF SPAWNING BEHAVIOUR IN THE MALE GOLDFISH** submitted by **ANN L. KYLE** in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

Abstract

When lesions were stereotaxically placed in medial nuclei of the ventral telencephalon and preoptic area of male goldfish, only lesions in the *area ventralis telencephali pars supracommissuralis* (Vs) and posterior *area ventralis telencephali pars ventralis* (pVv) (regions that bind sex steroids) reduced the proportion of males spawning both 5 days and up to 4 weeks postoperatively, as compared to sham groups. Subsequent experiments utilizing Vs–pVv lesioned fish showed that spawning consistency over 7 weekly tests was negatively correlated with the volume of Vs–pVv destruction. The motor capability for spawning behaviour was not affected, as two-thirds of Vs–pVv lesioned males spawned on at least one of their weekly tests, with latencies for the onset of each courtship behaviour similar to those of control fish. Neither was the reproductive endocrinology appreciably altered by Vs–pVv lesions, as the gonadosomatic indexes and "Con A II" gonadotropin (GtH) levels were similar in lesioned and sham lesioned goldfish.

Vs–pVv lesioned males that failed to show male spawning behaviour also failed to show female spawning behaviour when injected with prostaglandin F_2alpha (PG). When fish were temporarily isolated, less swimming activity was seen in the sexually inactive lesioned fish than in the controls. All behaviour was not indiscriminantly reduced, however, as feeding behaviour evoked by a food odor was unaffected by Vs–pVv lesions. The lesions may have disrupted the processing of stimuli that regulate social activities, particularly, for male behaviour, the processing of critical olfactory cues. In this regard, parallels with the mammalian amygdala were noted.

While the systemic implantation of testosterone (T) pellets reinstated spawning behaviour in sexually inactive, regressed male goldfish, other routes of androgen administration failed to significantly increase the proportion of males spawning. The males used in these latter experiments may have been rendered insensitive to androgen treatment by lengthy exposure to conditions that produced extreme sexual regression. Trends in these data suggested that systemically injected 11-ketotestosterone was more effective than T or estradiol in inducing male spawning. The observations that Vs–pVv implants of T pellets into regressed fish increased, while implants of the antisteroid drug, enclomiphene citrate, decreased this behaviour are consistent with the hypothesis of a

central site of androgen action.

When mature male goldfish were exposed to sexual stimuli (either a spawning PG-treated female or male) the levels of GtH and expressible milt were elevated within 1 h; these elevations persisted for at least 2 and 24 h respectively. These responses did not occur if the males were sexually inactive or physically separated from a stimulus spawning pair. The results indicated that exposure to a spawning situation was sufficient to induce these physiological changes, and that sexual behaviour and elevations in milt and GtH may share a common activational pathway. A combination of neural and hormonal mechanisms may mediate the sexually-stimulated increase in milt volumes.

Acknowledgments

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I. GENERAL INTRODUCTION

"Psychoneuroendocrinology" has been defined as a field that "covers both the control of hormonal function by the nervous system and the role of hormones themselves on brain function and behavior" (Smythies, 1976). This thesis represents the first concerted effort to advance our knowledge of the psychoneuroendocrinology of spawning behaviour in male fish.

In nonpiscine male vertebrates, particularly mammals and birds, these interrelationships have been well described. With the approach of the breeding season, environmental stimuli act via the hypothalamus to increase the secretion of pituitary gonadotropins, which stimulate spermatogenesis and the production of testicular androgens. These androgens are taken up by specific sites in the central nervous system that coordinate aspects of reproductive function. Classical castration/hormone replacement experiments have shown that sexual behaviour, as well as other processes such as the development of secondary sexual characteristics, are dependent upon adequate levels of circulating androgens. Furthermore, implanting small hormone pellets directly into the preoptic area of the hypothalamus, one of the sex steroid-concentrating brain sites, selectively activates mating behaviour without stimulating other androgen-dependent events. This suggests that androgens somehow "turn on" specific parts of the brain, which then facilitate sexual behaviour in response to the appropriate external stimuli. In turn, the performance of sexual behaviour causes short-term increases in gonadotropins and androgens, and while the functional significance of this response is still unclear, it presumably acts to facilitate reproductive processes that are rate-limited by hormone availability. Thus, the foregoing events ensure that sexual behaviour will occur when gonad maturation is complete (Martin, 1976; Kelly and Pfaff, 1978; Leshner, 1978; Larsson, 1979).

Little parallel information exists for fish. The teleost fishes appear to follow the general vertebrate pattern in terms of their reproductive endocrinology; environmental factors cue the onset of gonadal recrudescence through the elevation of gonadotropin and sex steroid hormones (Peter and Crim, 1979). Sex steroid binding sites have been identified autoradiographically in the ventral telencephalon, preoptic and tuberal hypothalamus, and pituitary of four species of teleosts, although the functional significance of

steroid binding has been directly studied only in the two latter areas, which are involved in mediating the feedback effects of steroids (Peter, 1982). However, the preoptic hypothalamus has been implicated in the control of gonadotropin secretion (Peter and Paulencu, 1980) and some aspects of male reproductive behaviour (Macey et al., 1974; Satou et al., 1980; Demski and Hornby, 1982); the function of the ventral telencephalon in steroid-dependent events is unknown. While castration/hormone replacement studies generally agree that male spawning behaviour in fish is steroid-dependent (Liley, 1969), there have been no attempts to stimulate sexual behaviour by implanting steroids into the brain. Also, nothing is known about alterations in endocrine state as a result of spawning activity.

For a psychoneuroendocrine study, the male goldfish (*Carassius auratus*) has several advantages as the experimental animal of choice:

1. it is an easily available, reasonably hardy animal that is bred for laboratory use;
2. much is known about its reproductive endocrinology;
3. it is one of the few fish for which exists an assay for gonadotropin and a stereotaxic atlas of the telencephalon and hypothalamus;
4. sexually active males can be produced throughout the year by manipulation of the photoperiod and temperature as well as by treatment with human chorionic gonadotropin (Yamamoto and Yamazaki, 1966). Females are normally receptive only when ovulated, but qualitatively normal female sexual behaviour can be elicited in fish in almost any reproductive state by treatment with prostaglandin F_2alpha (PG) (Stacey, 1976; Stacey and Peter, 1979). Furthermore, male goldfish do not discriminate between ovulated and PG-treated females (Stacey, 1981).

This thesis consists of four main studies, each presented as a self-contained paper. Chapter II describes the first experiments on the effects of brain lesions on spawning behaviour in the male goldfish. The experiments of Chapter III measured other behavioural parameters in male goldfish that were rendered sexually inactive by brain lesions. These results suggested ways in which lesions might be disrupting male spawning behaviour and pointed to possible homologies of the lesion site with parts of the mammalian brain. Preliminary studies, reported in Chapter IV, examined the effect of systemic steroid treatment on the behaviour of sexually regressed male goldfish and

attempted to manipulate the occurrence of spawning behaviour by implanting steroids or antisteroid drugs into the brain. Finally, Chapt. V gives the first report of rapid changes in the physiology of male goldfish due to the performance of sexual activity.

II. EFFECTS OF FOREBRAIN LESIONS ON SPAWNING BEHAVIOUR IN THE MALE GOLDFISH

A. INTRODUCTION

Relatively little is known about the neural substrates controlling male spawning behaviour in fish. In other vertebrates, central nervous sites that are major integrative areas for the potentiation of male sexual behaviour are activated following the binding of sex steroid hormones, thus ensuring synchrony between physiological and behavioural readiness for reproduction. Several brain areas that concentrate sex steroid hormones have been autoradiographically identified in four species of teleosts (Davis et al., 1977; Demski, 1978; Kim et al., 1978a; 1979). The tuberal hypothalamus, preoptic area, and ventral telencephalon were the most consistently identified sites, a pattern observed throughout the vertebrates (Morrell et al., 1975; Kim et al., 1978b). The stimulatory effects of systemically administered androgens on the spawning behaviour of male teleost fishes (Liley, 1969; Johns and Liley, 1970; Fernald, 1976; Villars and Davis, 1977) suggests that some of these sex steroid-concentrating brain areas may play an important role in reproductive behaviour.

Two sex steroid-concentrating areas, the preoptic area and telencephalon, have been implicated in the control of reproductive behaviour in fish. Preoptic electrical stimulation of male sunfish (*Lepomis*) evoked some components of reproductive behaviour (Demski and Knigge, 1971; Demski, 1978) and central nervous stimulation of sunfish and goldfish defined a sperm release pathway running from the preoptic area to the rostral spinal cord (Demski et al., 1975; Demski and Hornby, 1982). Large lesions of the *nucleus preopticus* blocked the neurohypophysial hormone-induced "spawning reflex" of killifish (*Fundulus heteroclitus*) (Macey et al., 1974), although the hormonal site of action is probably not in the brain (Peter, 1977; Pickford et al., 1980). It should also be noted that neurohypophysial hormones produce a "spawning reflex" only in cyprinodonts, and the relevance of this induced reflex to the natural sequence of spawning behaviour is unclear (Stacey, 1981). Medial preoptic lesions in male salmon (*Oncorhynchus nerka*) reduced the courtship behaviour directed towards a female, but did not reduce spawning (Satou et al., 1980). The preceding information and the involvement

of the medial preoptic area and anterior hypothalamus in the regulation of male sexual behaviour in mammals (Larsson, 1979), birds (Hutchison, 1976), reptiles (Wheeler and Crews, 1978; Morgantaler and Crews, 1978; Crews and Morgentaler, 1979), and amphibians (Schmidt, 1973; Wada and Gorbman, 1977a; 1977b), have led to the suggestion that the preoptic area may be a homologous neuroanatomical substrate for male sexual behaviour in all vertebrates (Kelly and Pfaff, 1978; Morgantaler and Crews, 1978).

Ablation of the telencephalon causes major deficiencies in spawning and other behaviours of many teleosts (Davis et al., 1976; de Bruin, 1980). The elimination of spawning by telencephalon ablation in the male paradise fish (*Macropodus opercularis*) was not due to perceptual or motor deficiencies, incompatible social responses (Kassel et al., 1976), or lowered nonspecific social arousal, suggesting that the telencephalon specifically facilitates the activation of reproductive behaviour (Kassel and Davis, 1977). Moreover, localized lesions of the dorsal telencephalon, which appear not to have destroyed the more ventral sex steroid-concentrating cells, were relatively ineffective in reducing sexual behaviour when compared to total telencephalon ablation in sticklebacks (*Gasterosteus aculeatus*) (Segaar and Nieuwenhuys, 1963) and Siamese fighting fish (*Betta splendens*) (de Bruin, 1977).

The present study examines the effects on spawning behaviour of lesions placed stereotaxically in and adjacent to the sex steroid-concentrating areas of the ventral telencephalon and preoptic area of the male goldfish (*Carassius auratus*). Preliminary results of this study have been reported (Kyle and Peter, 1978).

B. METHODS

Animals and Maintenance

Goldfish were purchased from Grassyfork Fisheries Co., Inc., Martinsville, Indiana and kept for various times in 1000 or 3500 litre flowing-water tanks, at 13 ± 1°C, under a simulated (Edmonton) photoperiod. Mature male goldfish, identified by the presence of pectoral tubercles and expressible milt, were moved to 150 or 225 litre flowing-water tanks and held at 20 ± 1°C, under a 16 h light and 8 h dark photoperiod, for at least two weeks before and during all experiments. Fish were fed twice daily with commercial fish

food (Ewos pellets).

Behavioural Testing Procedures

Production of Stimulus Females

Normally, spawning behaviour in the goldfish follows ovulation in the spring; however, as difficulty was experienced in producing sufficient numbers of ovulated females throughout the year, we routinely used intramuscular injections of prostaglandin F_2alpha (PG; 200 ng/g body weight) to produce sexually active females (Stacey, 1976; Stacey and Peter, 1979). Stacey (1981) has shown that male goldfish do not discriminate between ovulated and PG-treated females.

Preoperative Selection of Males

Groups of no more than three male goldfish were placed in 65 litre glass aquaria at 20°C that contained several bundles of floating, artificial plants, which served as a spawning substrate, and one or two sexually receptive females, injected 30 min previously with PG. Only animals that showed vigorous courtship activity within 30-40 min were selected for use in subsequent experiments.

Postoperative Tests

Experimental male goldfish were placed individually in aquaria containing a single PG-injected female and artificial plants, as described above, and were observed until spawning was seen or for 90 min. If no courtship activity was seen within 60 min, a stimulus male that was known to be sexually active was added to the aquarium, allowing courtship and spawning to occur during the last 30 min of the test. Spawning of the stimulus male and female served to verify the receptive state of the female and provided conditions that are often a strong courtship incitement for male goldfish (A.L. Kyle, unpublished observations). The onset latencies for three aspects of courtship behaviour, defined below, and spawning were recorded to the nearest min. Following the behavioural test, fish were returned to the holding tank; only at this time was the identity of each animal recorded.

Behavioural Categories

The following four components of male sexual behaviour, modified from Partridge et al. (1976) were defined: *following, quivering, rising,* and *spawning. Following* is a persistent orientation of the male towards the female, usually with his

head positioned against the female's ovipore; it often involves rapid pursuit of the female and is interspersed with bouts of *quivering*. *Quivering* consists of rapid, laterally vibrating movements of the male's body against the side of the female, which bring the tubercles of the operculum and pectoral fins into contact with her side and belly. *Rising* and *spawning* behaviours are initiated by the receptive female, who approaches the floating vegetation in a characteristic head-up posture. If the male accompanies her, then a *rise* is scored. *Spawning* proceeds from the *rise* position; the pair roll over on their sides and describe an arc through the water, with the male always positioned on the inside of the curve, underneath the female. At the peak of the arc, a vigorous flip of their tails coincides with egg and milt release, although the release of the latter cannot easily be seen. During the *spawning* of an unovulated, PG-injected female, eggs are, of course, not released.

Lesioning Procedure

Electrodes, made of size 0 insect pins insulated to within 0.5-0.6 mm of the tip, were stereotaxically positioned in various medial nuclei of the ventral telencephalon or preoptic area, using the method described by Peter and Gill (1975). Brain lesions were made with a Grass radiofrequency lesion-maker, model LM4, set at 90 (exp. 2.1) or 65 (exp. 2.2) volts for 30 sec. Sham lesions were produced in an identical manner, except that no current was applied to the electrode. Sham operations consisted of exposure of the brain surface without placement of the electrode. All surgery was done under 0.1% tricane methanesulfonate anesthesia. Animals were identified by a numbered tag attached to the operculum. The average weights (mean ± S.E.) were 28.7 ± 0.7 g for fish in exp. 2.1 and 36.2 ± 1.4 g for those in exp. 2.2.

Tissue Samples

At the end of each experiment, fish were anesthetized and killed; brains were removed for histological analyses and testes for determination of the gonadosomatic index (GSI=gonad weight x 100/total body weight). Brains were fixed in Bouin's solution, embedded in paraffin, sectioned at a thickness of 7 *u*m, stained with paraldehyde fuchsin, and counterstained with acid fuchsin, Ponceau xylidine, and fast-green. The perimeter of the lesioned area for each fish was drawn on a reference series of cross-sectional brain outlines taken from the atlas of Peter and Gill (1975). The percentage of the nuclear

volume destroyed was estimated by calculating the ratio of the lesioned area within a specific nucleus to the total area occupied by that nucleus on the reference series of brain outlines.

Exp. 2.1 - Forebrain Lesions

One or two days after their preoperative behavioural test, sexually active male goldfish were assigned to one of three treatment categories: brain lesion, sham lesion, or sham operation, and the appropriate surgery was performed. A total of five different electrode locations were used, two in the preoptic area and three in the ventral telencephalon. The fish were retested for spawning behaviour five days postoperatively and killed two days later for removal of brains and testes. This protocol was followed for 8 groups of 10-15 fish, from October until early February. Each group included lesion and sham treatments for two of the five electrode locations, and each electrode location was repeated across two or three groups to increase the sample size. Data from any animal whose lesion either failed to be formed or was excessively large or misplaced were excluded from subsequent analyses. The final sample sizes were 38 brain lesioned, 34 sham lesioned, and 10 sham operated male goldfish.

Exp. 2.2 - Lesions in the Supracommissural Telencephalon

To test the hypothesis, generated by exp. 2.1, that an intact supracommissural telencephalon (Vs-pVv) is required for the normal expression of male spawning behaviour in goldfish, sexually active males were either brain lesioned (n=21) or sham lesioned (n=10) in this area. Disturbance of the anterior commissure and secondary damage to the dorsal telencephalon were avoided by the use of modified electrode coordinates and a lower lesioning voltage (65v), as compared to exp. 2.1 (90v). The fish were retested for spawning behaviour 1, 2, 3, 4, and 7 weeks postoperatively, although not all animals were tested on weeks 4 and 7; this gave a total of 135 animal-tests. After the final behavioural test for each group, the animals were killed and the brains and testes removed. Data from any fish that became ill during the course of the experiment were excluded. This experiment extended from January to April.

C. RESULTS

Exp. 2.1 - Forebrain Lesions

From the results of their postoperative behavioural test, all but one fish could be readily assigned to either "spawning" or "nonresponding" categories, as they either displayed the full sequence of courtship and spawning activity, or failed to respond to the stimulus female. The exception, a sham lesioned male that displayed vigorous courtship behaviour but failed to *rise* or *spawn*, was placed in the "spawning" category. Exposure to a stimulus spawning pair during the last 30 min of the test induced spawning in only two previously unresponsive animals.

The effect of brain lesions on the ratio of spawning:nonresponding males was examined in two ways. In the first analysis, animals were grouped together if at least 25% of the volume of a particular nucleus was destroyed, although the lesions need not have destroyed a similar region within that nucleus. If a lesion destroyed at least 25% of two (or more) nuclei, then that fish was assigned to two (or more) groups. For each nucleus, the spawning:nonresponding ratio for brain lesioned males was compared to that ratio for the appropriate sham lesioned group (Fisher's exact test, one-tailed). As shown in Fig. 2.1, lesions in the *area ventralis telencephali pars ventralis* (Vv; p=0.002) or the *area ventralis telencephali pars supracommissuralis* (Vs; p=0.07) reduced the percentage of spawning males, while lesions in the *area ventralis telencephali pars postcommissuralis* (Vp), *nucleus preopticus periventricularis* (NPP), or *nucleus preopticus* (NPO) had no effect. The percentages for the sham operated and sham lesioned groups were not different.

.A second analysis was done to localize the effective area more precisely within the nuclei and to incorporate data from animals with lesions destroying less than 25% of any nucleus. In this case, a grid was superimposed upon a near-midsagittal outline of the goldfish forebrain, with each grid-unit representing a 53x53 *u*m square of tissue along the sagittal plane. For each grid-unit, the ratio of spawning:nonresponding males was calculated for the group of fish containing lesions in that area and tested against the spawning:nonresponding ratio of the sham operated controls (Fisher's exact test, one-tailed). In this way, probability values for the equality of the two ratios were generated across the forebrain grid. The results (Fig. 2.2) show that lesions in the Vs and

only the posterior portion of the Vv (pVv) significantly reduced the number of males spawning.

Sexually active brain lesioned and sham lesioned fish of any nuclear category did not differ in either the latency to the onset of *following* or the intervals between *following* and the three other behaviours. Furthermore, although this was not experimentally tested, lesioned males did not appear to differ obviously from sham lesioned or sham operated males in other aspects of their behaviour, such as feeding, startle response, and general swimming activity.

Mean GSI values for the 8 groups increased from 2.03 in early October to 4.17 in February. Because not all fish assigned to the same nuclear category came from the same experimental group, GSI values within and between nuclear categories could not be meaningfully compared. However, there was no difference between the GSIs of spawning and nonresponding males within each experimental group. For the sexually active animals, there was no correlation between GSI and the latency to any activity.

A problem in the interpretation of this experiment is that secondary damage to the dorsal telencephalon or interruption of fibres of the anterior commissure that occassionally occurred as a result of some of the lesions may have contributed to the observed behavioural deficiencies. This problem was circumvented by the procedural changes of exp. 2.2.

Exp. 2.2 - Lesions in the Supracommissural Telencephalon

As in exp. 2.1, most of the behavioural tests (122/135) of the lesioned and sham lesioned fish could be readily assigned to either "spawning" or "nonresponding" categories. Of the 13 tests where animals showed partial responsiveness, there were 9 designated as "courting only", where only *following* and *quivering* occurred, and 4 designated as "nonresponding", where only fragmentary *following* was seen, such as that which often occurs in a nonsexual context.

Fig. 2.3 shows that there was a reduction lasting 3 weeks in the proportion of lesioned males showing at least *following* and *quivering* and a 4 week reduction in the proportion of those showing the complete spawning sequence, as compared to sham lesioned controls (Fisher's exact test, one-tailed, $p<0.05$). On the seventh week, these two proportions are neither statistically different from the sham value for that week or

Fig. 2.1. Percentage of male goldfish spawning that were either sham operated (n=10), sham lesioned (n=34), or lesioned (n=27) in various brain nuclei. (n) – number of fish with ≥25% of the nucleus destroyed. p – calculated by Fisher's exact test, one-tailed, only significant values shown. NPO – *nucleus preopticus*; NPP – *nucleus preopticus periventricularis*; Vp – *area ventralis telencephali pars postcommissuralis*; Vs – *area ventralis telencephali pars supracommissuralis*; Vv – *area ventralis telencephali pars ventralis*.

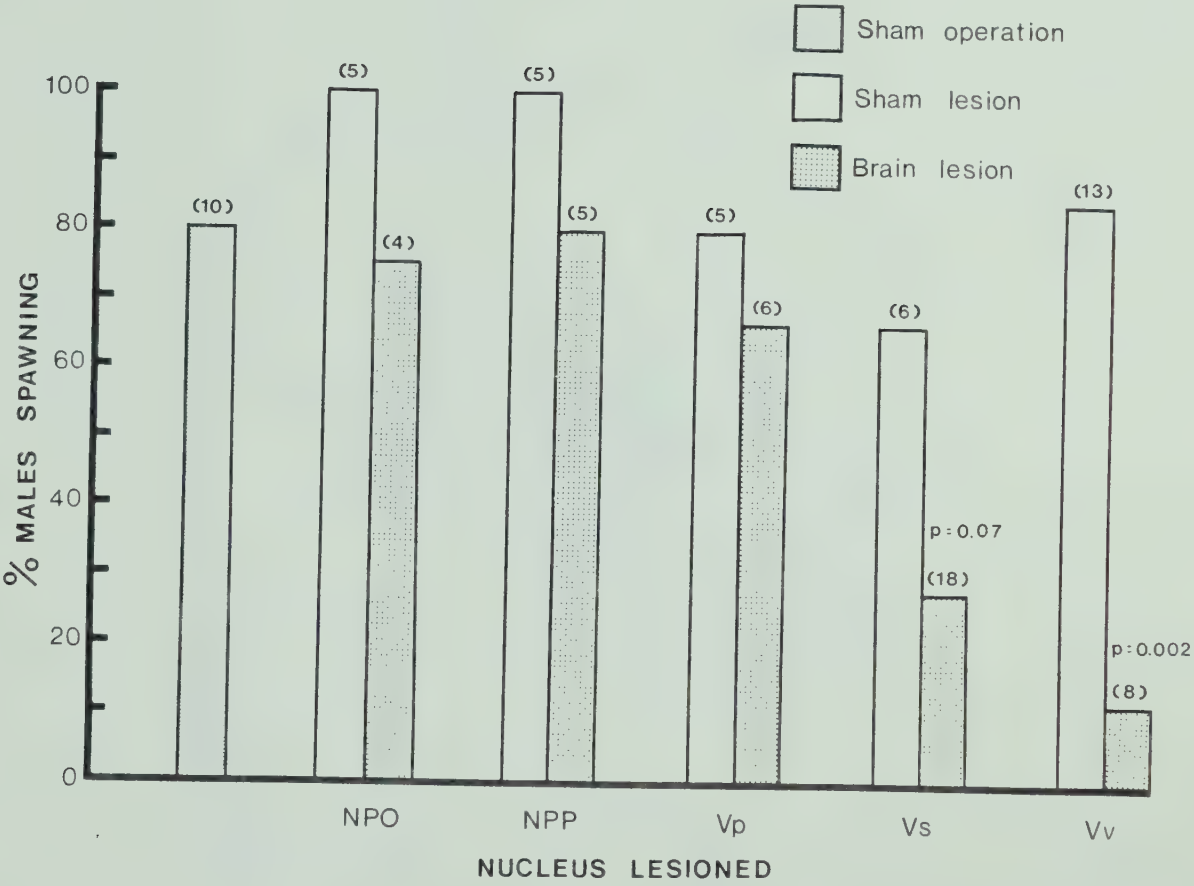
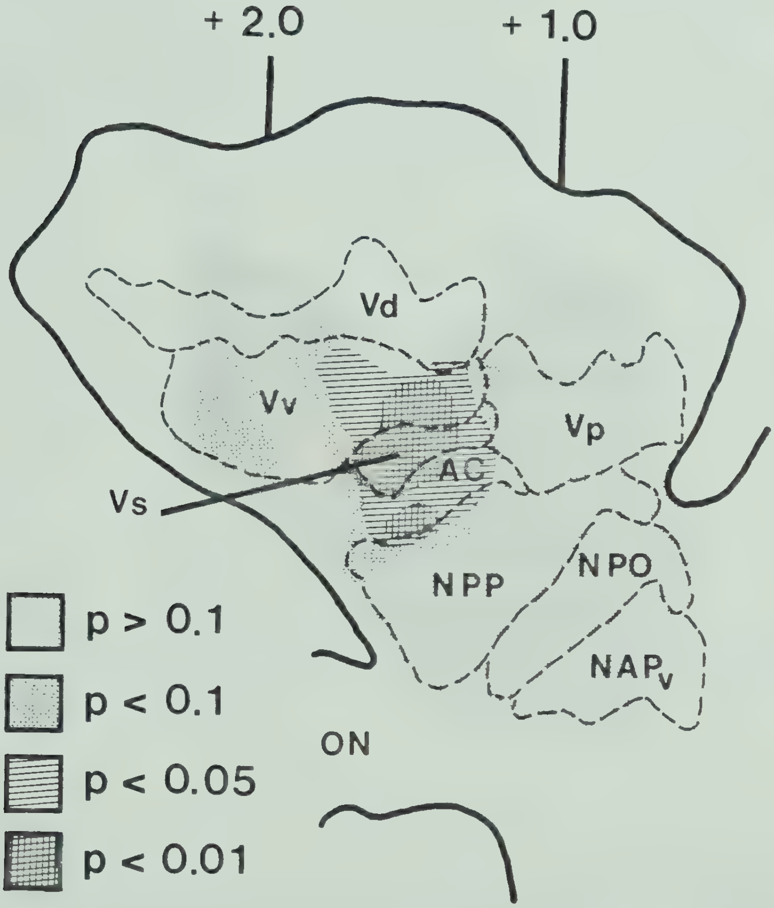


Fig. 2.2. Parasagittal outline of the goldfish brain 50 *um* lateral to the midline; numbers above the drawing refer to the atlas of Peter and Gill (1975). Shading indicates brain areas that, when lesioned, have equal probabilities of reducing the proportion of spawning males relative to sham operated controls, as calculated by Fisher's exact test, one-tailed. Analysis based on 38 lesioned and 10 sham operated males. AC – anterior commissure; NAPv – *nucleus anterioris periventricularis*; ON – optic nerve; Vd – *area ventralis telencephali pars dorsalis*; other abbreviations as in previous figure.



the lesioned values for previous weeks.

Fish were not always consistent in their behaviour from test to test; of the 21 lesioned animals, 6 failed to spawn on any test, 3 spawned on every test, and the remaining 12 spawned on some tests but not others. In Fig. 2.4, the percentage of tests in which an individual performed the complete spawning sequence is plotted against the volume of the target area lesioned (Vs–pVv, as defined in Fig. 2.2); sham lesioned animals were included as those having 0% volume of this area destroyed. A negative correlation (Spearman's rank correlation, one-tailed, $p<0.001$) was found between the volume of the Vs–pVv area lesioned and spawning consistency. The lesion locations of the most severely impaired animals, those which failed to spawn on all or all but one of their tests, are shown in Fig. 2.5 and can be seen to involve the supracommissural portion of the ventral telencephalon that contains the Vs, posterior Vv, and adjacent parts of the *area ventralis telencephali pars dorsalis* (Vd). Fig. 2.6 is a photomicrograph of a lesion that destroyed most of the Vs and parts of the pVv. The less severely impaired fish generally had smaller or dorsally displaced lesions, which resulted in less destruction of the target area.

As in exp. 2.1, the latency to the onset of *following* and the interval between *following* and *quivering*, *rising*, or *spawning* were not different between sexually responding lesioned and sham lesioned fish on any week. The GSI values were also unaffected by brain lesioning, being (mean ± S.E.) 3.56 ± 0.38 for the lesioned fish and 3.52 ± 0.60 for the shams.

D. DISCUSSION

Exp. 2.1 examined the effects of lesions placed in various medial ventral telencephalic and preoptic nuclei on the spawning behaviour of male goldfish. Two separate criteria were used to assign fish to lesion treatment groups: similarity of the nucleus into which the lesion fell, which assumes functional uniformity within a nucleus (Fig. 2.1) and similarity of position, which is independent of any anatomically defined boundaries (Fig. 2.2). The former analysis found that only lesions in the Vv or Vs reduced the proportion of spawning males, while the latter identified just the supracommissural portion of the ventral telencephalon (Vs–pVv), an area known to concentrate ^{3}H estradiol

Fig. 2.3. Percentage of sham lesioned (S) or Vs–posterior Vv (Vs–pVv) lesioned (L) male goldfish showing either the complete spawning sequence or only courtship behaviour, at weekly intervals after surgery. (n) – sample size. p – calculated by Fisher's exact test, one–tailed.

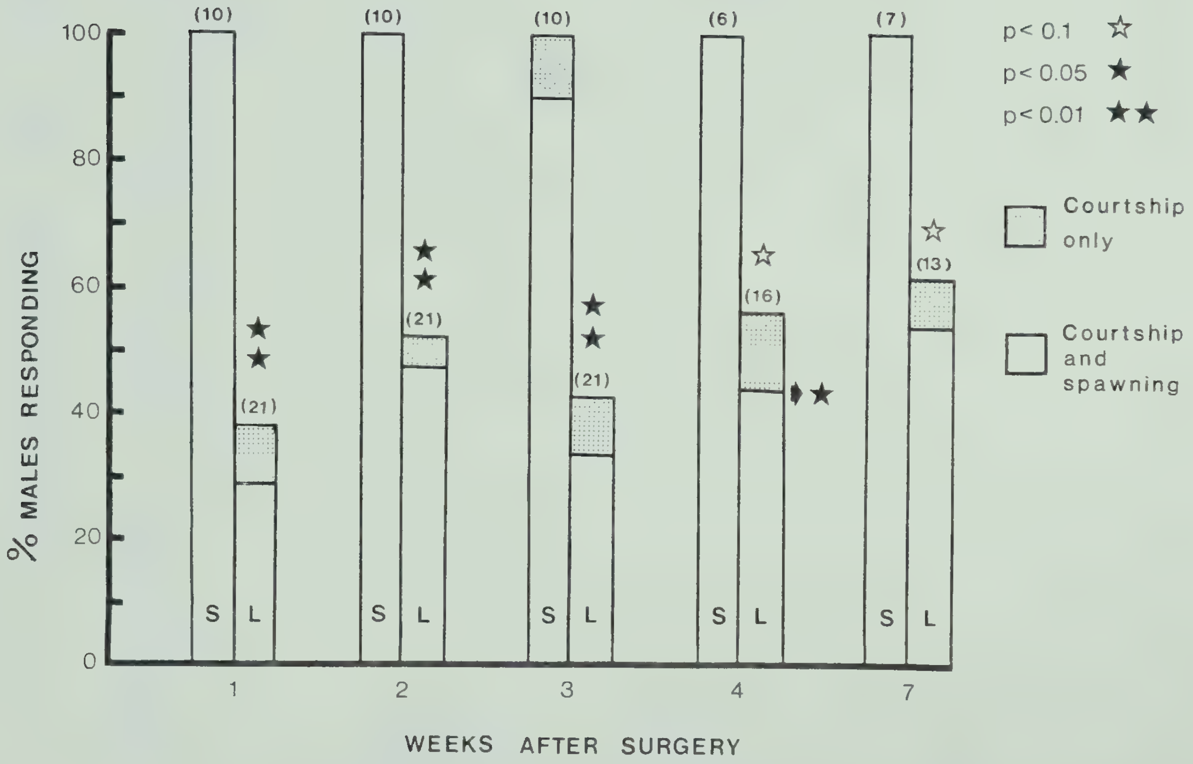


Fig. 2.4. Correlation between the percentage of tests in which spawning occurred and the volume of the Vs–pVv area destroyed. Rs – Spearman's rank correlation coefficient, one-tailed test.

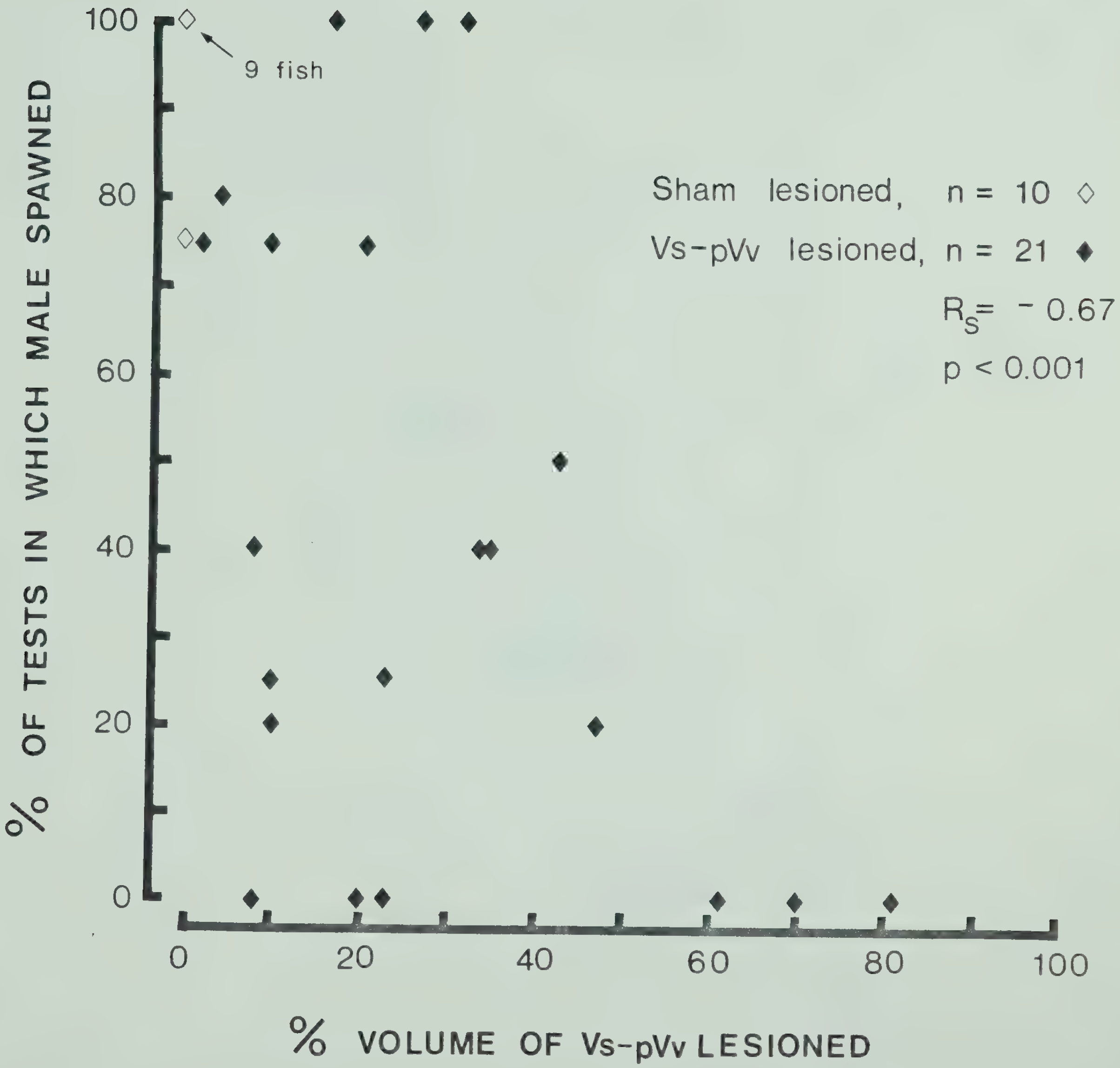


Fig. 2.5. Outlines of the extent of the lesioned areas in fish with severe spawning impairment, n=10, as determined by weekly spawning tests. The distance between each cross-section is 0.2 mm; numbers above each drawing refer to standard brain outlines adapted from the atlas of Peter and Gill (1975). OC – optic chiasma; Vl – *area ventralis telencephali pars lateralis*; other abbreviations as in previous figures.

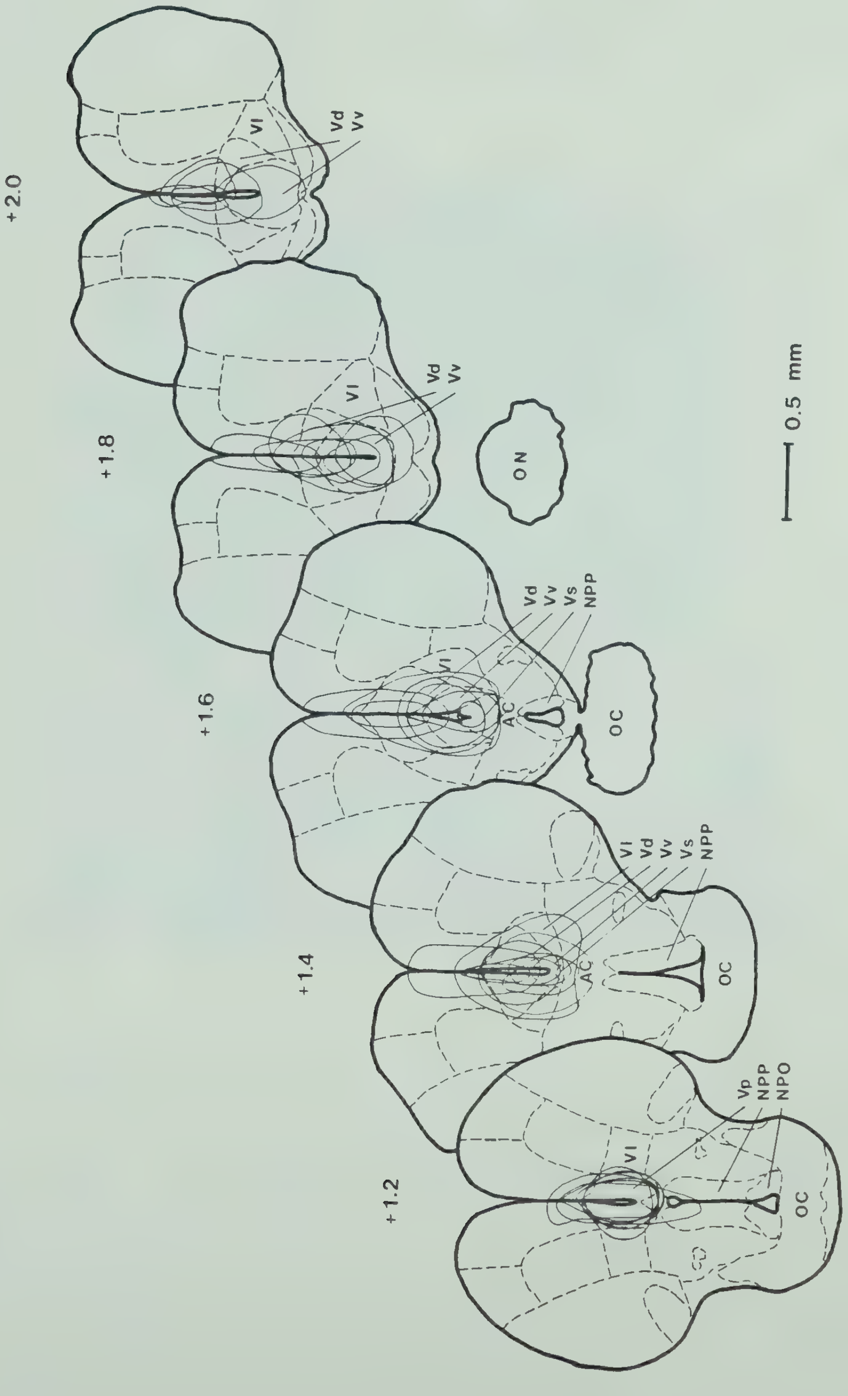
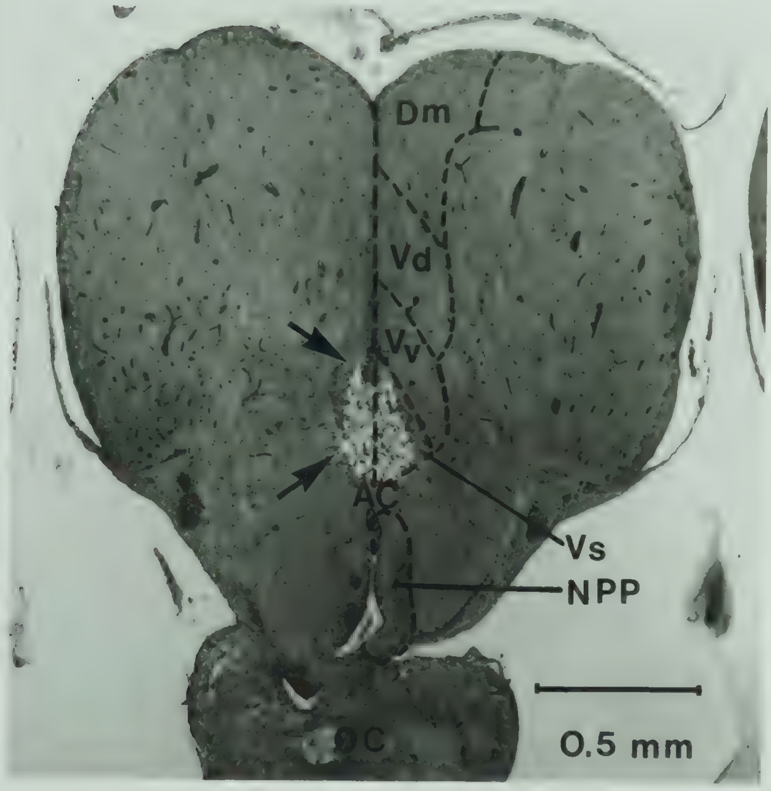


Fig. 2.6. Photomicrograph taken at the anterior–posterior coordinate of approximately +1.6 (Peter and Gill, 1975) of a supracommissural lesion in the goldfish telencephalon. Arrows indicate the lesion site. Dm – *area dorsalis telencephali pars medialis*; other abbreviations as in previous figures.



in goldfish (Kim et al., 1978a), as the effective region.

The lack of effect of preoptic lesions does not negate a role for this site in spawning behaviour. No lesion of the NPP or NPO destroyed more than half of the nucleus; perhaps larger lesions would have had an effect. Few of these lesions overlapped the same space, creating small sample sizes in the preoptic grid-squares used to calculate Fig. 2.2 and, therefore, a high chance of accepting a false null hypothesis. Similarly, if the assumption behind Fig. 2.1 (uniformity of nuclear function) was not met, then the apparent ineffectiveness of NPP or NPO lesions may be because the appropriate subregion within the nucleus was rarely destroyed. However, indirect support for the assumption of functional uniformity is provided by the observations that the effects of lesions on the spawning reflex of killifish (Macey et al., 1974) or the mating behaviour of male rats (Larsson, 1979) could not be attributed to the destruction of any particular subregion of the preoptic nucleus. It may be, as Demski and Hornby (1982) have suggested, that the preoptic area controls the reflexive aspects of spawning, such as sperm release, while the supracommissural telencephalon is involved in mediating sexual arousal. In contrast to this suggestion, preoptic lesions in salmon impaired sexual arousal (as measured by the occurrence of quivering) without affecting "orgasm" (Satou et al., 1980), and preoptic stimulation of rainbow trout (*Salmo gairdneri*) failed to evoke sperm release (Demski and Hornby, 1982). This suggests that the Salmonidae may differ from goldfish in the function of the preoptic area in reproductive behaviour.

In exp. 2.2, Vs-pVv lesions reduced the proportion of fish spawning for at least four weeks postoperatively. The negative correlation between consistency of spawning and lesion size and the fact that even relatively small lesion volumes resulted in fish with spawning behaviour deficiencies suggests that the supracommissural portion of the telencephalon is a critical area for spawning regulation. There may have been some behavioural recovery on week 7, although the smaller size of the sham lesion group on weeks 4 and 7 makes such a conclusion tentative. Behavioural recovery may be contingent upon neuronal reconstruction or functional alterations in the remaining tissue (Kassel and Davis, 1977). In goldfish, ablated portions of the telencephalon do not regenerate, although some tracts are restored following stab wounds (Bernstein, 1967).

This study does not provide any information regarding the mechanism of action of Vs–pVv lesions in blocking spawning behaviour. Gonadosomatic index measurements indicated that some factor other than testicular condition accounted for the differences in behaviour between brain lesioned and sham lesioned or sham operated fish, although testes weight may not have reflected changes in androgen or gonadotropin levels. It is also unlikely that the lesions destroyed the motor capabilities for spawning, as two–thirds of the lesioned males in exp. 2.2 performed all components of spawning behaviour on at least one of their weekly tests. The finding that either the full sequence or complete absence of the spawning sequence was seen for the majority of lesioned animals, suggests that the lesions resulted in a failure to initiate courtship, perhaps by causing a lowered arousal for general activity or specifically for reproductive behaviour and its activating stimuli.

Aronson and Kaplan (1968) and Aronson (1970) proposed that the teleost telencephalon functions as a nonspecific arouser of behaviour organized elsewhere in the brain. While this hypothesis may account for many of the behavioural deficiencies of telencephalon ablated fish, it does not predict that differential decrements in various reproductive behaviours would occur (Segaar and Nieuwenhuys, 1963; Ribbink, 1972) or that some social behaviours would be unimpaired following telencephalon ablation (Aronson, 1948; Kamrin and Aronson, 1954; Kassel and Davis, 1977). Although no nonsexual behaviours were measured in our study, the fact that the latency parameters for courtship and spawning recorded during the 90 min test were not different between responding lesioned males and controls suggests that supracommissural telencephalic lesions disrupted spawning behaviour specifically, rather than depressing the general activity seen under these experimental conditions (but see Chapt. III).

Telencephalic lesions may interfere with spawning behaviour by impairing the perception of sexual cues. For example, telencephalon ablated male cichlids (*Hemihaplochromis philander*) were unable to visually distinguish between males and females (Ribbink, 1972), and, although earlier experiments failed to find any components of spawning behaviour in telencephalon ablated male paradise fish (Davis et al., 1976; Kassel et al., 1976; Schwagmeyer et al., 1977), some of these activities were seen in later studies that used females showing a higher intensity of prespawning behaviour

towards the male (Kassel and Davis, 1977). In the goldfish, where anosmia results in a marked decrease in male sexual behaviour, it is thought that a pheromone released by ovulated females acts as an important stimulant for male courtship (Partridge et al., 1976). Also, olfactory tract section reduced male courtship of unovulated, PG-treated females, suggesting that PG-treatment renders a fish sexually attractive by inducing the appropriate odor, as well as the behaviour (N.E. Stacey and A.L. Kyle, unpublished results). Since reciprocal connections exist between the olfactory bulbs and ventral telencephalon in goldfish (Oka, 1980), supracommissural telencephalic lesions may have reduced male courtship of PG-treated females by disrupting the processing and relaying of critical olfactory information. In this regard, the supracommissural telencephalon may be analogous to the medial portion of the corticomedial amygdala of rodents, which is an androgen-binding brain area that receives input from the vomeronasal organ via the accessory olfactory bulb and projects to the medial preoptic-anterior hypothalamic area (Scalia and Winans, 1975). In hamsters, perception of sexual chemosensory information and performance of male sexual behaviour depends upon the integrity of this system, as vomeronasal deafferentiation (Powers and Winans, 1975) or lesions of the medial amygdaloid nucleus (Lehman et al., 1980) resulted in severe mating deficiencies.

The Vs-pVv of teleosts is usually regarded as a septal, rather than an amygdaloid homologue, although the location proposed for the latter varies with the species and the researcher (Droogleever Fortuyn, 1961; Nieuwenhuys, 1967; Northcutt and Braford, 1980) and the situation in goldfish has not been addressed specifically. If lesions in the Vs-pVv reduce male sexual behaviour of goldfish by reducing olfactory input, then it would be reasonable to propose a homology between all or part of the Vs-pVv and the corticomedial amygdala.

While disruption of olfactory processing may have contributed to the spawning impairment seen after brain lesioning, we also propose that the supracommissural telencephalon (Vs-pVv) has a more complex function than simply relaying olfactory information. For example, whereas PG-induced spawning behaviour in female goldfish is relatively unaffected by olfactory tract section, Vs-pVv lesions reduced sexual behaviour in females (A.L. Kyle and N.E. Stacey, unpublished results) as well as in males. This demonstrates that the Vs-pVv plays an important role in the spawning behaviour of both

sexes, even though only the behaviour of the male is heavily dependent upon olfactory input.

III. VENTRAL TELENCEPHALIC LESIONS: EFFECTS ON BISEXUAL BEHAVIOUR, ACTIVITY, AND OLFACTION IN THE MALE GOLDFISH

A. INTRODUCTION

Although a large body of literature is devoted to describing the behavioural consequences of telencephalic ablation in fish, little is known about the influence of specific telencephalic areas on specific types of behaviour (de Bruin, 1980). In the male goldfish (*Carassius auratus*), discrete lesions in the ventral telencephalon just dorsal and anterior to the anterior commissure, the *area ventralis telencephali pars supra-commissuralis* and posterior part of the *area ventralis telencephali pars ventralis* (Vs–pVv), severely impair male spawning behaviour (Chapt. II); however, the way in which these lesions produce this behavioural deficiency is unknown.

Since the Vs–pVv concentrates sex steroids (Kim et al., 1978a) and male goldfish spawning behaviour is steroid-dependent (Chapt. IV), this region may be important in the control of male sexual behaviour. Vs–pVv lesions may interfere with the perception and processing of relevant sexual cues and/or the output of information to other brain areas involved in the execution of behaviour. In particular, these lesions may interfere with the perception of olfactory cues, probably in the form of pheromones released by receptive females (Partridge et al., 1976), as portions of the ventral telencephalon have reciprocal connections with the olfactory bulbs (Oka, 1980) and section of the olfactory tracts also impairs sexual behaviour in male goldfish (Partridge et al., 1976; N.E. Stacey and A.L. Kyle, unpublished results). Alternatively, Vs–pVv lesions may function in a less specific manner, by reducing responsiveness in a social or general context. Ablation of the entire telencephalon impairs the performance of such species-specific behaviours as aggression, nest building, courtship, spawning, and parental care (de Bruin, 1980; Laming and McKee, 1981), as well as some aspects of general activity and learning (Flood et al., 1976; Savage, 1980). A satisfactory hypothesis has yet to be proposed that will account for this syndrome (Savage, 1980); however, even relatively restricted lesions of the telencephalon may affect processes that underlie a variety of behaviours.

This study attempted to discover the way in which lesions of the Vs–pVv result in decreased male sexual behaviour by measuring other behavioural parameters in lesioned

male goldfish. The question of whether Vs-pVv lesions are specific in disturbing only one type of social interaction was addressed by examining both male and female sexual behaviour in the same individual; qualitatively normal female behaviour can be induced in males by prostaglandin F_2alpha (PG) treatment (Stacey, 1981). The possibility that failure to show sexual responsiveness is correlated with abnormal activity or impaired olfactory perception also was examined. Finally, testes weights and serum gonadotropin levels were measured to determine whether the lesions act by disrupting the hypo-thalamo-pituitary-gonadal axis.

B. METHODS

Animals and Maintenance

Fifty male goldfish, with tubercles on the pectoral fins and operculum and expressible milt, were selected from stocks obtained from the Grassyforks Fisheries Co., Inc., Martinsville, Indiana, housed in two 225 litre flowing-water tanks at 20°C under a natural simulated (Edmonton) photoperiod, and fed twice daily with commercial food (Ewos pellets). Fish were kept under these conditions one month before and between the behavioural tests (June and July), but were not fed for five days prior to the food odor tests.

Day 0 - Brain Lesions

Fish were anesthetized in a 0.05% solution of 2-phenoxyethanol (Syndel) and lesioned in the Vs-pVv using the method of Peter and Gill (1975). A 0.3 mm diameter electrode, insulated to within 0.5 mm of the tip, was positioned at coordinates +1.4, M, D 1.2 and a radiofrequency current of 70 volts was applied for 30 sec using a Grass lesion maker, model LM4. Sham lesions were produced in an identical manner, except that no current was applied to the electrode. Stitches were removed on day 10.

Day 11 - Male Behaviour Tests

All sexual behaviour tests were done in 65 litre glass observation aquaria that contained coarse aquarium gravel, subsand filters, and floating, artificial plants. Each aquarium was painted on three sides, illuminated with a single fluorescent fixture mounted 20 cm above the water surface, and arranged in a darkened, quiet room. Prior to the testing of the experimental males, receptivity was induced in stimulus females by

the intramuscular (i.m.) injection of PG at 200 ng/g body weight. (Stacey, 1976; Stacey and Peter, 1979) and verified by checking that each female spawned with a sexually active "stud" male. The stud males then were removed, experimental males were added individually to the observation aquaria, and the amount of time that each male spent courting or spawning was recorded with a Rustrak event recorder for 60 min. A description of male goldfish courtship and spawning behaviour is given elsewhere (Chapt. II).

Day 15 - Female Behaviour Tests

PG treatment can also induce males to perform female spawning behaviour that is essentially indistinguishable from that performed by females, with maximum activity occurring about 45 min after injection (Stacey, 1981). A female spawning act is initiated with a rise into the floating vegetation. Accompanied by the male, the fish performing the female role (either a female or a PG-treated male) turns on its side and swims an arc-like path through the water, always positioned on the outside of the arc above the male. Ovulated eggs, if present, are released simultaneously with a flip of the tail at the arc apex.

On day 13, pairs of mature male goldfish were moved into the observation aquaria and a PG-injected female was added to each aquarium on the morning of day 15. The female and one of the two males were later removed, retaining the more sexually active male for use as a stud. Each experimental male was added individually to the observation aquarium one hour after the i.m. injection of 250 ng PG/g body weight, and female spawning acts were recorded for 60 min.

Day 18 - Activity and Food Odor Perception Tests

In preparation for this test, food was withheld from day 13. A random subset of sham lesioned fish and those Vs-pVv lesioned fish that failed to respond on both their male and female sexual behaviour tests were placed individually in 65 litre glass aquaria on the evening of day 17. These aquaria were similar to the sexual behaviour observation aquaria, except that the non-opaque side was curtained and grid lines were drawn on the glass; a small flap cut in the curtain could be raised for observations. For the first 10 min of the test, the number of grid-lines crossed and spontaneous bites at the gravel substrate were recorded for each fish. Then, 0.5 ml of a 2% aqueous solution

of "Tetramin" brand fish food was added into the tank via a 1 cc syringe fitted with a 15 cm length of polyethylene tubing; in most cases, this could be done without disturbing the test fish. This food extract had been prepared previously by grinding Tetramin flakes in distilled water into a slurry with a mortar and pestle, centrifuging the slurry, and freezing the supernatant for later use. Immediately after the introduction of the food extract into the aquarium, the number of times that the test fish mouthed the substrate was recorded for a further 10 min. Sham and brain lesioned males were tested alternately throughout the day to mask any possible daily activity rhythms.

Day 19 - Blood and Tissue Samples

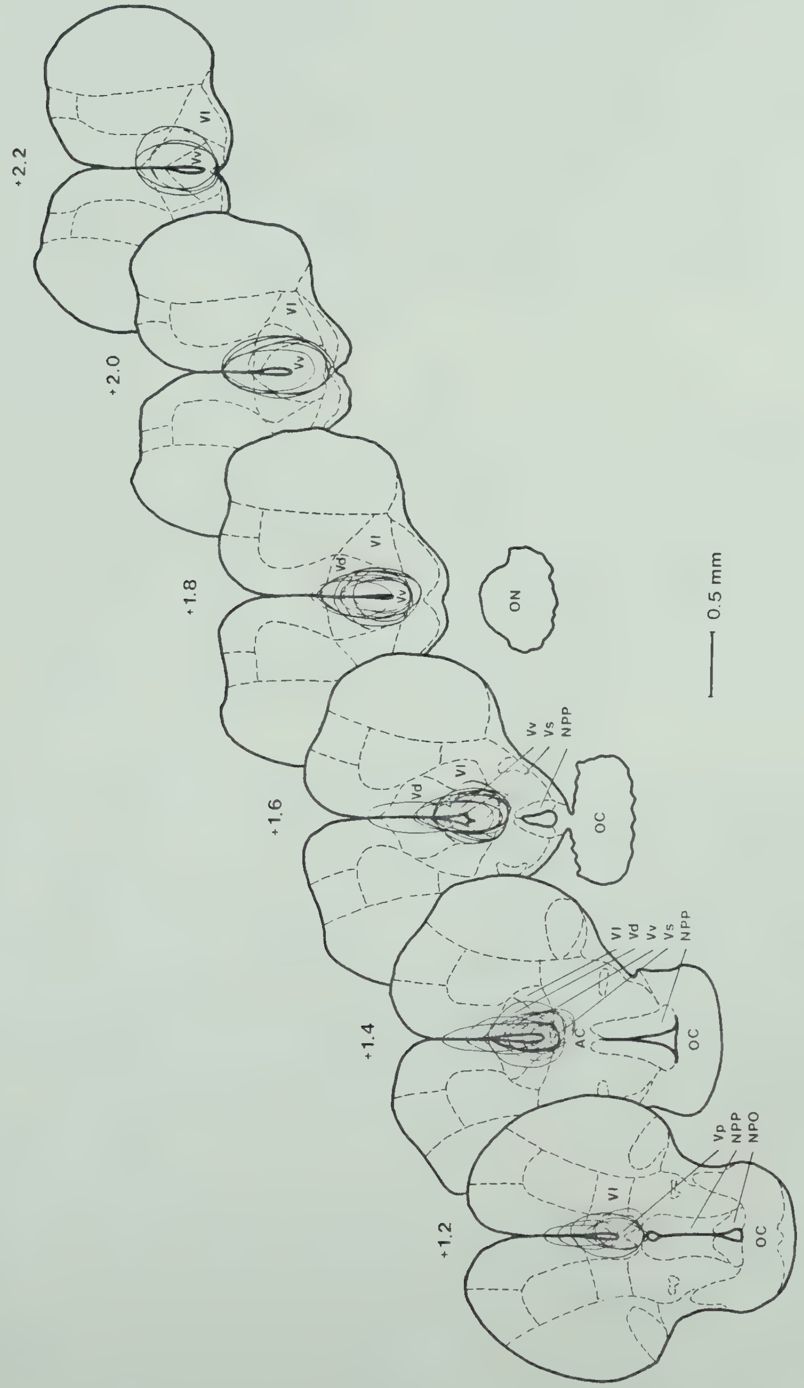
Fish were anesthetized and bled via puncture of the caudal vasculature in the afternoon. After clotting on ice, the blood was centrifuged at 5°C, and the serum was collected, quick-frozen, and stored at -28°C. Serum GtH levels were determined by radioimmunoassay (Crim et al., 1976; Hontela and Peter, 1978; 1980).

While still under anesthesia, animals were killed by decapitation, and the brains and testes were removed. Gonadosomatic indexes were calculated by the formula: (GSI=gonad weight x 100/total body weight). Brains were fixed in Bouin's solution, embedded in paraffin, sectioned at a thickness of 7 *u*m, stained with paraldehyde fuchsin, and counterstained with acid fuchsin, Ponceau xylidine, and fast-green. The perimeter of the lesioned area for each animal was drawn on a reference series of cross-sectional brain outlines taken from the atlas of Peter and Gill (1975). Estimates were made of both the total size of each lesion and the volume of the Vs-pVv destroyed.

C. RESULTS

As was seen in previous work (Chapt. II), lesions that were placed too far ventrally interrupted the anterior commissure and adjacent vasculature and caused degeneration of other areas of the telencephalon. In the following analyses, only animals with lesions within the Vs-pVv area and with no obvious telencephalic degeneration are considered. These lesion positions are given in Fig. 3.1 and can be seen to encompass most of the Vs-pVv and extend dorsally into the *area ventralis telencephali pars dorsalis*. Lesions destroyed an average of 30% (5-80% range) of the Vs-pVv area.

Fig. 3.1. Extent of telencephalic lesions in male goldfish, n=17. The distance between each cross-section is 0.2 mm; numbers above each drawning refer to standard brain outlines adapted from the atlas of Peter and Gill (1975). AC – anterior commissure; NPO – *nucleus preopticus*; NPP – *nucleus preopticus periventricularis*; OC – optic chiasma; ON – optic nerve; Vd – *area ventralis telencephali pars dorsalis*; Vl – *area ventralis telencephali pars lateralis*; Vp – *area ventralis telencephali pars postcommissuralis*; Vs – *area ventralis telencephali pars supracommissuralis*; Vv – *area ventralis telencephali pars ventralis*.



Male Behaviour

Vs–pVv lesions were effective in reducing the number of fish spawning (Table 3.1, $p<0.001$, Fisher's exact test, one-tailed) and the total time spent courting (Fig. 3.2, $p<0.001$, Mann–Whitney U test), as compared to the sham treatment. Fig. 3.2 also shows that the amount of time that the sham fish spent courting increased over the 60 min observation period. This was due to both the increase in the number of animals initiating courtship, which occurred mainly in the first 30 min, and the increase in the courtship time scored for each individual. About half of the lesioned fish showed some courtship activity. Like the sham males, this activity was initiated within 30 min, but unlike the sham males, the individual courtship times did not increase during the test. Neither the total lesion size nor the volume of the Vs–pVv area destroyed differed between courting and the nonresponding fish.

Female Behaviour

Half of the sham males, but only one of the lesioned males, showed female spawning behaviour when injected with PG (Table 3.1, $p<0.01$, Fisher's exact test, one-tailed). The total number of female spawning acts for this lesioned fish (7/60 min) was below the range for the sham animals (8–28/60 min). Any fish, whether sham or lesioned, that responded as a female also had responded as a male on day 11.

Activity

The lesioned males used in this test had failed to respond on both the male and female behaviour tests and also were noticeably less active when isolated than were the sham males tested (Table 3.1, $p=0.02$, Mann–Whitney U test, two-tailed). Typically, sham fish constantly "fluttered" up and down one side of the aquarium, while lesioned animals tended to intersperse bouts of slow swimming throughout the tank with periods of inactivity. All animals appeared healthy and alert, with the fins held erect and away from the sides of the body.

Food Odor Perception

Within 5 sec of odorant addition, most fish responded with an "alert" posture, a spreading of the fins and momentary cessation of movement. This was followed by rapid swimming over the bottom of the tank and mouthing of gravel particles. Although the number of bites performed after odor addition varied considerably between

Fig. 3.2. Amount of time that sham or Vs–pVv lesioned male goldfish spent courting a female. The means for each 10 min interval are shown on the graph and the histogram gives the medians and ranges for the entire 60 min test. p – calculated by Mann–Whitney U test, one–tailed.

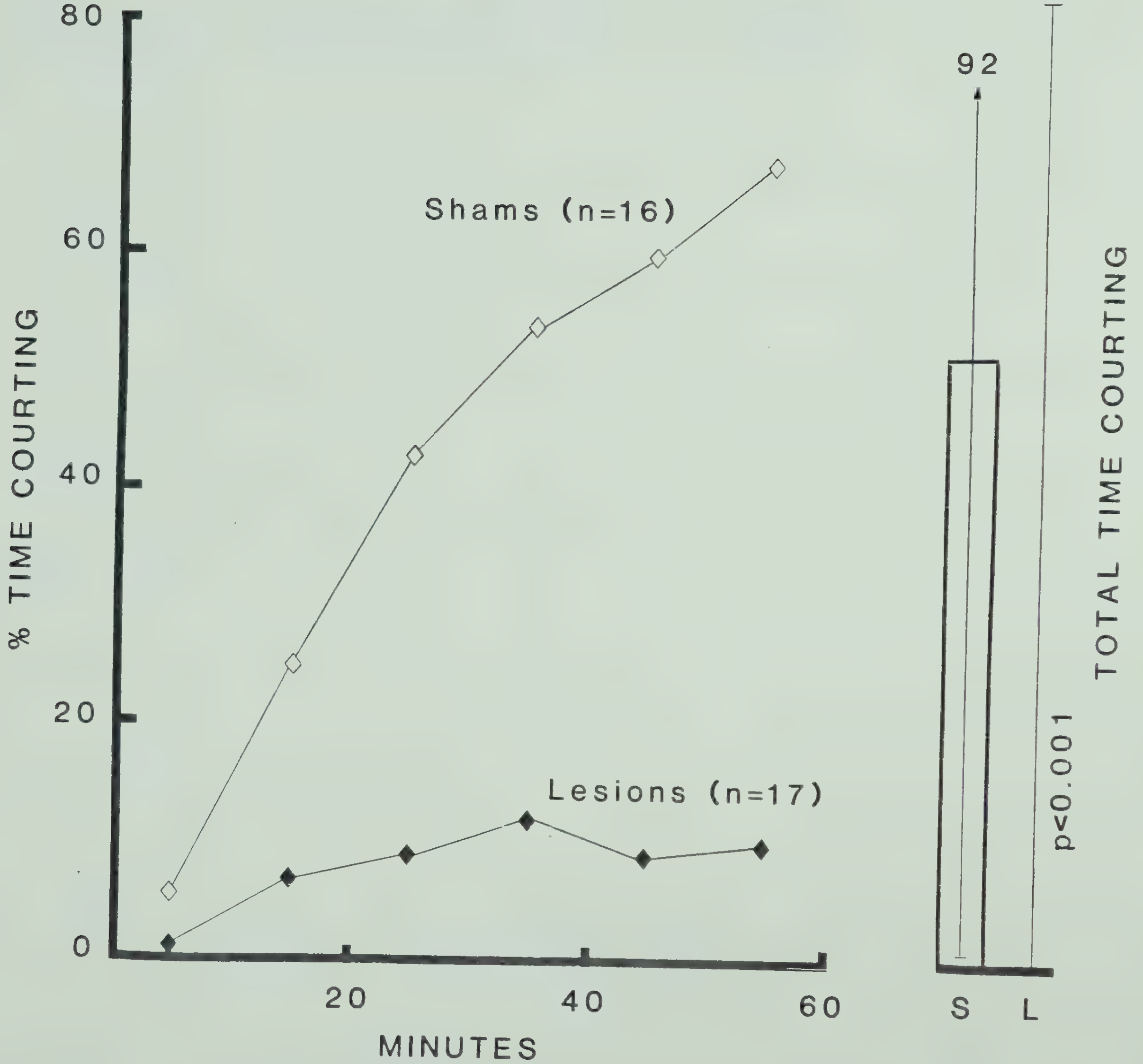


Table 3.1. Effects of Vs–pVv lesions on various physical and behavioural parameters in male goldfish.

	SHAMS	LESIONS	P
Sample size	16	17	
GSI◇	3.16 ± 0.33	3.06 ± 0.26	n.s.▽
GtH (ng/ml)◇	14.17 ± 1.50	13.16 ± 1.19	n.s.▽
% spawning as males	88	18	$p<0.001$*
% spawning as females	50	6	$p<0.01$*
Sample size	12	8	
Activity(squares crossed)◆	156 (15-365)	25 (0-127)	$p=0.02$▼
% responding to food odor	75	63	n.s.*

◇ mean ± S.E.

◆ median (range)

▽ t-test, 2-tailed

* Fisher's exact test, 1-tailed

▼ Mann-Whitney U test, 2-tailed

individuals, the temporary increase of activity and mouthing of the gravel clearly indicated that the food odor had been perceived. Neither the proportion of fish responding to the food odor (Table 3.1, Fisher's exact test, one-tailed) nor the number of pre- or post-odorant bites (two-tailed t-test) differed between sham and lesioned groups.

GSI and Gonadotropin

Neither the GSI values nor the plasma GtH concentrations differed between the sham and Vs-pVv lesioned males (Table 3.1, t-test, two-tailed). Body weights measured at the end of the experiment also were not different.

D. DISCUSSION

The results of this study support the previous finding that lesions of the supra-commissural telencephalon (Vs-pVv; an area including the *area ventralis telencephali pars supracommissuralis*, the posterior portion of the *area ventralis telencephali pars ventralis*, and the adjacent posterio-ventral *area ventralis telencephali pars dorsalis*) reduce male spawning behaviour in the goldfish (Kyle and Peter, 1978; Chapt. II) and provide information regarding the way in which these lesions might be exerting their behavioural effect.

Both the present and previous studies produced lesions that destroyed a similar volume (about 30%) of the Vs-pVv and found a similar proportion (about 50%) of lesioned males initiating courtship one week postoperatively. However, in the previous study, almost all of the lesioned males that courted females eventually spawned, while in the present work, only about one-third of the lesioned, courting males spawned. It is unlikely that the longer test time used in the earlier work (90 vs. 60 min) accounted for this difference *per se*, as almost all the courting males spawned within one hour. However, the males of the earlier study had been given a preoperative sexual behaviour test, whereas the males in the present work were not given prior exposure to the test situation. Possibly this prior experience increased the liklihood of spawning in the males of the first study.

A loss of olfactory function has been suggested as a mechanism by which Vs-pVv lesions might depress male sexual behaviour (Introduction, Chapt. II). The olfactory capability of lesioned fish was assessed by observing the occurrence of

feeding behaviour evoked as a result of exposure to a food extract. This is an olfactory-dependent response, as it is reduced by plugging the olfactory pits (Grimm, 1960) or sectioning the olfactory tracts (Westerman and von Baumgarten, 1964; N.E. Stacey, D. Goulet, and A.L. Kyle, unpublished results). The proposed role of the telencephalon in food extract-induced feeding is to process the olfactory information and relay it to areas in the hypothalamus that organize the feeding movements (Peter, 1979). The response to a food odor was not impaired in Vs-pVv lesioned animals, although further testing with more dilute solutions of food extract may have revealed threshold differences; however, other information raises the question of whether other categories of odorants, such as pheromones, can be perceived.

The bilateral olfactory tracts of goldfish each have medial (m.o.t.) and lateral (l.o.t.) subdivisions. Experiments involving section of either the m.o.t. or l.o.t. revealed that the reduced courtship seen in completely olfactory tract-sectioned males (Partridge et al., 1976; N.E. Stacey and A.L. Kyle, unpublished results) was due to the loss of the m.o.t. (N.E. Stacey and A.L. Kyle, unpublished results). As only the m.o.t. projects to the supracommissural telencephalon of goldfish (Oka et al., 1982), Vs-pVv lesions may reduce male sexual behaviour by disrupting the perception of pheromonal odors. In contrast, both the m.o.t. and the l.o.t. were involved in mediating the feeding response to a food odor (N.E. Stacey, D. Goulet, and A.L. Kyle, unpublished results), indicating that Vs-pVv lesions would be unlikely to completely impair food odor perception.

The possibility that pheromonal perception may be impaired in Vs-pVv lesioned males only explains the reduction of male behaviour. PG-induced female spawning behaviour, which is not dependent upon olfactory input (N.E. Stacey and A.L. Kyle, unpublished results), is also reduced by Vs-pVv lesions in both males (Table 3.1) and females (A.L. Kyle and N.E. Stacey, unpublished results), suggesting that these lesions depress general sexual performance. Almost no other information has been reported concerning the central neural control of female spawning behaviour in fish. Noble (1936) observed that the movements of egg laying and fertilization in cichlids depended upon "part of the posterior tubercle", a term that perhaps was intended to describe the ventral telencephalon and/or preoptic area (de Bruin, 1980). In female goldfish, injection of PG into the third ventricle of the brain was more effective than the same dose given

peripherally (Stacey and Peter, 1979). Taken together, these results suggest that the Vs-pVv is required for the induction of female sexual behaviour by PG. Whether the same or different areas within the Vs-pVv control male versus female spawning behaviour cannot be determined from this study.

When fish were temporarily isolated, less swimming activity was seen in the sexually inactive lesioned males as compared to the sham lesioned controls. The "arousal hypothesis" suggests that the various behavioural deficiencies incurred after telencephalon ablation are caused by the loss of nonspecific facilitation of lower brain areas (Aronson and Kaplan, 1968). Thus, this hypothesis would attribute the reduction in male and female sexual behaviour and activity to a general disinclination to respond to any stimulus. However, the results of the present experiment and other studies show that all behaviour is not indiscriminantly reduced. Feeding activity evoked in response to a food odor was unaffected by Vs-pVv lesions, both in the number of animals responding and in the intensity of the response. As well, even in goldfish with total telencephalic ablation, simple measures of arousal, such as bradycardia in response to a novel stimulus, and certain types of learning are unimpaired (Laming and McKee, 1981; Savage, 1980). It is not surprising that the arousal hypothesis cannot account for these results, since it considers the telencephalon as a functionally homogeneous unit, a concept incompatible with the morphological and biochemical regionalization of the telencephalic substructure.

Male and female spawning behaviour may be grouped together within the class of "species-specific" or "social" behaviour. Although swimming activity is not usually considered to be a social behaviour, it is highly dependent upon the social condition under which it is observed. For example, goldfish in groups showed a decrease in oxygen consumption and activity (Schuett, 1934; Shlaifer, 1938) and isolated fish reduced their activity when they were presented with their own mirror image (Shlaifer, 1939). In our laboratory, isolated goldfish are typically hyperactive and become more calm once placed with another fish (A.L. Kyle, unpublished observations). The decreased activity of isolated Vs-pVv lesioned fish, therefore, may be due to an insensitivity to social stimuli (or the absence thereof). Whether the activity of Vs-pVv lesioned fish kept in groups is similar to that of sham animals is not known.

If Vs–pVv lesions disrupted GtH regulatory systems, then both sexual behaviour and activity could be affected in the goldfish, as activity (Hoar et al., 1955) and male behaviour (Chapt. IV) are facilitated by androgens and the spawning response to PG treatment in females apparently requires priming by steroids (Stacey and Liley, 1974) and/or GtH (Stacey, 1976). Some evidence suggests that the telencephalon may play a modulatory role in the regulation of GtH secretion; telencephalon ablated male paradise fish (*Macropodus opercularis*) showed a significant decrease in GSI as well as in male sexual behaviour (Kassel et al., 1976) and gonadotropin releasing factor has been demonstrated immunohistochemically in telencephalic areas as well as in the hypothalamus (Goos and Murathanoglu, 1977; Münz et al., 1981). However, the present finding that both GtH concentrations and GSIs were similar in lesioned and control fish suggests that impairment of the pituitary–gonadal axis is not a mechanism by which Vs–pVv lesions cause their behavioural effects.

A number of parallels suggest that the Vs–pVv of goldfish and the corticomedial amygdala of mammals play a similar role in the regulation of male sexual behaviour:

1. lesions in both areas cause deficiencies in male behaviour (Chapt. II – goldfish; Lehman et al., 1980 – hamsters);
2. both areas receive input from specific olfactory tracts (Oka et al., 1982 – goldfish; Scalia and Winans, 1975 – mammals), and section of these olfactory tracts results in sexual behaviour deficiencies (N.E. Stacey and A.L. Kyle, unpublished results – goldfish; Powers and Winans, 1975 – hamsters); and
3. both areas project to the preoptic area (Schroeder, 1980 – teleosts; Kevetter and Winans, 1981 – hamsters), a site also involved in male sexual behaviour in vertebrates (Kelly and Pfaff, 1978; Demski and Hornby, 1982).

Can parallels also be found between other behavioural deficiencies caused by Vs–pVv lesions in goldfish and amygdaloid lesions (AL) in mammals? The effect of AL on female sexual behaviour is variable. Lesions of the medial amygdala blocked receptivity in female deermice without altering the estrous cycle (Eleftheriou and Zolovick, 1966). In rats, corticomedial AL alone had no effect on receptivity, although they did attenuate the increased levels of receptive behaviour seen following septal lesions (McGinnis et al., 1978). In other mammals, receptivity remained unchanged or increased after AL

(Komisaruk, 1978). Similarly, different types of changes in locomotor activity following AL are described (Gloor, 1960), with at least one study reporting a permanent decrease in spontaneous activity (Anand and Brobeck, 1952). Olfactory discrimination of anise and food odors was retained in animals with bilateral AL (Swann, 1934; Allen, 1941), although the processing of pheromonal information is presumed to be a function of the cortico-medial amygdala (Lehman at al., 1980). Thus, while many similarities do exist between the behaviour of Vs-pVv lesioned goldfish and AL mammals, the variable responses of the latter confuse attempts at detailed comparisons.

Rather than examining specific studies that utilize AL, where the results likely vary with the species used, testing paradigm, and portion of the amygdala destroyed, a more fruitful approach may be to consider the overall effects of AL on behaviour. AL have been described as causing a reduction of social interactions (Kling, 1972), a lowered interest in the social signals sent by others of the same species (Isaacson, 1974), and a loss of a stimulus-analyzing system critical for the processing of salient cues that evoke species-specific behaviour (Cormier, 1981). These descriptions could also be applied to the Vs-pVv lesioned goldfish, and parallel the suggestion that these lesions decrease the sensitvity of the fish to social stimuli. Whether or not such parallels suggest homology between part or all of the Vs-pVv and the mammalian amygdala, analogies drawn between the ventral telencephalon of fish and the limbic system of mammals may provide a framework to assist the construction of meaningful hypotheses.

IV. EFFECTS OF SYSTEMICALLY OR CENTRALLY ADMINISTERED STEROIDS OR ANTISTEROIDS ON SPAWNING BEHAVIOUR IN THE MALE GOLDFISH: PRELIMINARY STUDIES

A. INTRODUCTION

Successful reproduction involves sequential synchronization of gonad maturation and sexual behaviour with those conditions appropriate for breeding. In most male vertebrates, synchrony between physiological and behavioural readiness for reproduction is maintained by testicular androgens, which are taken up by specific central nervous sites that control sexual behaviour. In fishes, the results of most castration and/or replacement therapy studies also indicate that gonadal androgen is the primary hormonal facilitator of male behaviour (Fernald, 1976; Johns and Liley, 1970; Liley, 1969; Reinboth, 1972; Villars and Davis, 1977), although the site of action is unknown. The present study examined the effects of systemically and centrally administered steroids or antisteroid drugs upon the spawning behaviour of the male goldfish (*Carassius auratus*).

There is little information regarding the behavioural endocrinology of goldfish. Pituitary hormones appear not to be necessary for the expression of spawning behaviour, as short-term hypophysectomized males were sexually active (Yamazaki, 1962). Also, methyl testosterone has been reported to induce courtship behaviour (Yamazaki, 1976), although there were no controls in that experiment and complete spawning behaviour could not be observed as the stimulus fish were unreceptive. Several sites in the teleost brain concentrate sex steroid hormones (Davis et al., 1977; Demski, 1978; Kim et al., 1978a; Kim et al., 1979) and lesion of one of these sites, the supracommissural telencephalon, blocked male spawning behaviour in goldfish (Kyle and Peter, 1978; Chapts. II and III); however, the behavioural effects of central steroid administration have not been investigated.

This study consisted of four parts. The first experiment examined the effects of systemic testosterone administration on the sexual behaviour of regressed male goldfish. The second employed systemic injections to compare the effectiveness of testosterone with some of its metabolites. The third attempted to induce sexual behaviour in regressed males by the stereotaxic implantation of testosterone pellets into the

supracommissural telencephalon, the *area ventralis telencephali pars supracommissuralis* and posterior *area ventralis telencephali pars ventralis* (Vs–pVv). Finally, as an alternate approach, the fourth experiment examined the possible inhibitory effect on the sexual behaviour of mature males of various antisteroid drugs implanted into several brain areas.

B. METHODS

Preparation of Sexually Inactive Male Goldfish

Castration of male goldfish was initially used in an attempt to produce sexually inactive animals. This technique was later discarded due to the high mortality, long recovery time, difficulty in removing all the testicular tissue, and eventual testicular regeneration; the latter two problems also have been encountered by others (Johns and Liley, 1970; Villars and Davis, 1977). Male goldfish are relatively reproductively regressed from September to December, when the lowest androgen levels (Schreck and Hopwood, 1974) and gonadosomatic index values (GSI=gonad weight x 100/total body weight) (Billard and Breton, 1978) are found. However, a significant proportion of males obtained in the winter from the Grassyfork Fisheries Co., Inc., Martinsville, Indiana, spawned and produced expressible milt when tested, making them unsuitable for this study.

In exps. 4.1, 4.2, and 4.3, the following procedure was adopted in an attempt to produce sexually inactive male goldfish. Regressed males, those that had little or no expressible milt and barely visible pectoral tubercles, were separated from autumn and winter stocks of goldfish and kept in 225 litre flowing–water tanks, at 30°C, under a 8 h light and 16 h dark (8L:16D) photoperiod, and were fed sparingly twice per week. These conditions were chosen to promote the regressed state, as a high temperature favours the formation of inactive androgen glucuronide conjugates (Kime, 1980; Kime and Saksena, 1980) and temporarily inhibits gametogenesis (Billard et al., 1978), while short photoperiods do not stimulate the hypothalamo–pituitary–gonadal axis (Peter and Hontela, 1978). After one to three months of this regimen, the fish were cooled to 20°C and individually placed in a 65 litre glass aquarium containing floating, artificial plants and a pair of actively spawning goldfish. The female of this spawning pair was made sexually

receptive by the intramuscular injection of 200 ng prostaglandin F_2alpha/g body weight (Stacey, 1976; Stacey and Peter, 1979). Male goldfish that showed no courtship activity during this 50 min pretest were moved to 150 litre flowing-water tanks, with a maximum of 25 fish/tank, at 20°C, 8L:16D, and the experimental protocol was begun on the next day. As will be described, this procedure for producing sexually inactive male goldfish was not entirely satisfactory.

Exp. 4.1 - Systemic Implants of Testosterone Pellets

As the duration of steroid treatment needed to induce a behavioural change in regressed goldfish was unknown, a sustained-release mode of testosterone (T) administration was chosen. T pellets were made by drawing a molten suspension of a 1:2 ratio by weight of T:beeswax into the barrel of a 1 cc syringe. Once cooled, the T:wax mixture was extruded in 0.5 cc volumes and cut into pellets weighing (mean ± S.E.) 48.97 ± 1.17 mg, resulting in an average T content per pellet of 16.3 mg. "Blank" pellets were made from beeswax alone.

Sexually inactive male goldfish, produced as described above, were anesthetized in a 0.1% solution of tricaine methanesulfonate and implanted intraperitoneally (i.p.) with either two T or beeswax pellets, by inserting the pellet through a small incision in the body wall that was then closed with a single suture. The fish weighed an average of 30.9 ± 2.7 g and were identified by fin clips. Animals were given a 60 min spawning test, as previously described (Chapt. II), 2, 5, and 40 days after implantation and were killed for GSI determinations and pellet recovery on the day after the last behavioural test. Some females were discovered at this time and were excluded from further analysis. All fish remained in good health throughout this experiment.

Exp. 4.2 - Systemic Injection of Various Steroids

Male goldfish, selected as described above, were numbered with opercular tags and injected i.p. with one of estradiol benzoate (EB), testosterone propionate (TP), or 11-ketotestosterone (11KT), at 7 ug/g body weight, or the vehicle (V) of 0.5% gelatin in physiological saline on alternate days for a total of 4 injections (7 days). The steroid and vehicle solutions were prepared by the method of Wiegand and Peter (1980) and were kept frozen between injections. Males were given a 60 min spawning test one and seven days following the last injection, and the occurrence of both courtship and spawning

activity was recorded. A description of male goldfish courtship and spawning behaviour is given elsewhere (Chapt. II). GSI determinations were made on the day following the last behavioural test. Because of the occurrence of bacterial disease and females mistakenly identified as regressed males, the sample sizes were reduced from 10 fish per treatment to 7 V, 5 EB, 9 11KT, and 6 TP treated males; the average body weight was 28.6 ± 1.4 g.

Exp. 4.3 - Brain Implants of Testosterone Pellets

Since exp. 4.1 was more effective than that of exp. 4.2 in inducing male spawning behaviour, T pellets were chosen for implantation into the Vs-pVv. Pellets were made from either a 1:2 by weight mixture of T:beeswax or from beeswax alone ("blank" pellets) and stereotaxically implanted as described by Billard and Peter (1977), using coordinates of +1.5, M, D 0.8 (Peter and Gill, 1975). These pellets weighed about 26 *ug*.

Sexually inactive males were anesthetized, indentified by fin clips, and implanted with either a T or blank pellet into the Vs-pVv. Fish were tested for spawning behaviour 4, 9, and 20 days after implantation and killed after the last behavioural test for GSI determinations and removal of brains for histology. The fish remained healthy throughout the experiment; however, due to a high proportion of females, almost 50%, final sample sizes were reduced to 6 fish for each treatment. The average body weight of these fish was 27.0 ± 1.9 g.

Exp. 4.4 - Brain Implants of Antisteroid Drugs

Although the protocol described under "Preparation of sexually inactive male goldfish" was followed, a proportion of the males in exps. 4.1-4.3 courted females on subsequent spawning tests, making comparisons between steroid and blank or vehicle treated groups difficult. In this experiment, the reverse approach was attempted of inhibiting the spawning behaviour in mature males by the use of antisteroid drugs. Drugs used were the antiandrogens cyproterone (Schering AG, Berlin), cyproterone acetate (Schering AG, Berlin), and flutamide (Schering Corp., Bloomfield), and the antiestrogens enclomiphene citrate (Merrell National Labs., Cincinnati), nitromifene citrate (Parke, Davis and Co., Detroit), and tamoxifen (Stuart Pharmaceuticals, Wilmington). Most pellets were a 30% by weight mixture of drug and cocoa butter, prepared as previously described (Billard and Peter, 1977), with "blank" pellets consisting of cocoa butter alone. Some

pellets contained 100% drug or cholesterol (blank); these were made by tamping the drug directly into the pellet holder. The pellet holder was stereotaxically inserted into various regions of the brain using the following coordinates: optic tectum (OT), +0.2, L 1.0, D 0.2; *nucleus preopticus* (NPO), +1.0, M, D 1.6; and the Vs-pVv, +1.4, M, D 0.9 (Peter and Gill, 1975).

Mature male goldfish, kept at 20°C and under a simulated natural (Edmonton) photoperiod, were pretested with a spawning pair as previously described. Males that showed courtship activity were anesthetized, fin clipped, and implanted with either one of six antisteroid drugs or blank pellets into the OT, NPO, or Vs-pVv. All combinations of drugs and brain areas were used, except that tamoxifen was only implanted in the Vs-pVv. The fish were given spawning behaviour tests several times during the first week and up to several weeks following implantation. After the last behavioural test, gonads were removed for GSI determinations and brains for histology; the average body weight was 31.1 ± 1.4 g. This experiment ran from April to July.

C. RESULTS

Exp. 4.1 - Systemic Implants of Testosterone Pellets

Fig. 4.1 shows that the proportion of T-treated males spawning was larger than that of the control group, both 2 ($p=0.07$; Fisher's exact test, one-tailed) and 5 ($p<0.001$) days after implantation. In fact, 100% of the T-treated males spawned on the fifth test day. By 40 days postimplantation, however, the T-treated males were less sexually active than on their previous tests, and the proportion spawning was not different from the controls. At the end of the experiment, the T-treated males had smaller GSIs than the controls, although the difference was not statistically significant.

Exp. 4.2 - Systemic Injection of Various Steroids

Injection of 7 *u*g/g body weight of either EB, 11KT, or TP had no stimulatory effect on male courtship behaviour (*following* and *quivering*) on either of the two test days (Fig. 4.2). More than half of the control group (V) courted females 7 days after treatment, although none of these fish spawned. The value most different from the controls was the proportion of 11KT-treated fish that spawned, as well as courted, on the seventh test day ($p=0.07$, Fisher's exact test, one-tailed). GSIs were not different

Fig. 4.1. Percentage of regressed male goldfish spawning when tested at various times after the i.p. implantation of either beeswax (B) or testosterone (T) pellets. (n) - sample size. p - calculated by Fisher's exact test, one-tailed. GSIs are not different, two-tailed t-test.

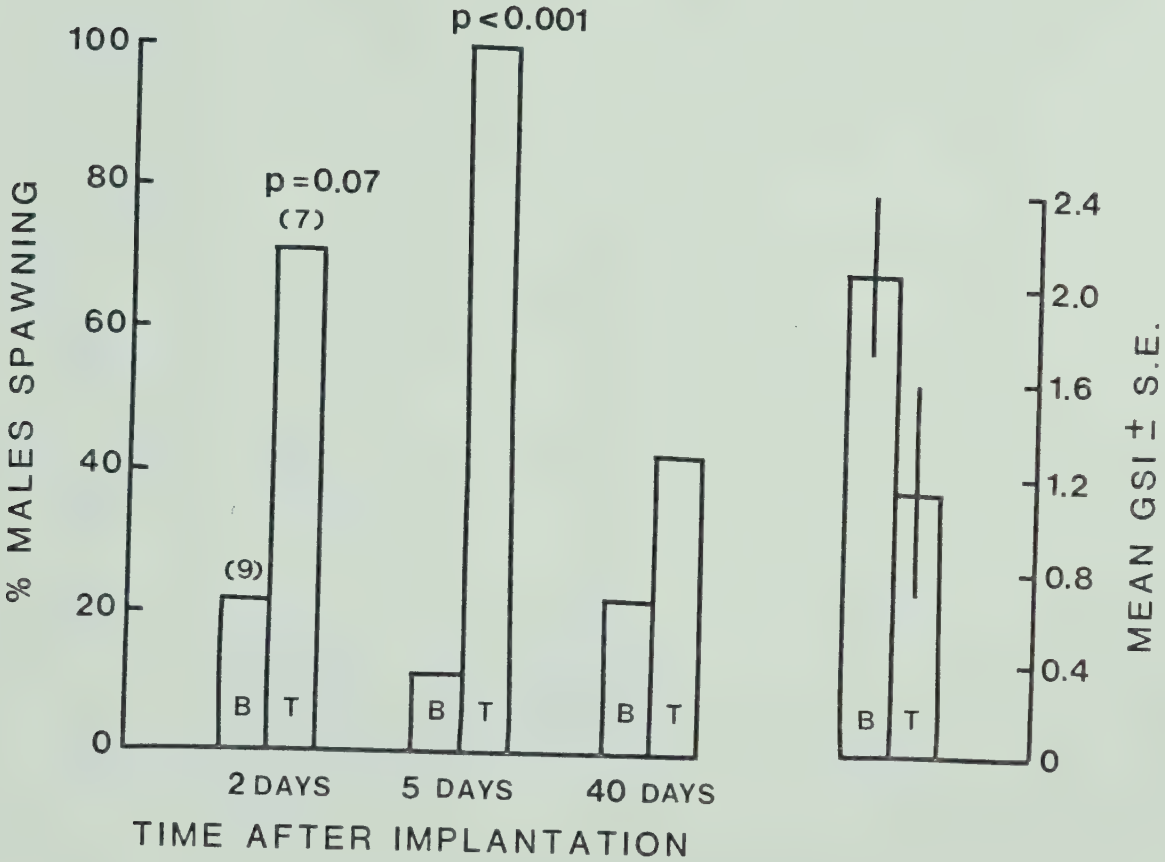
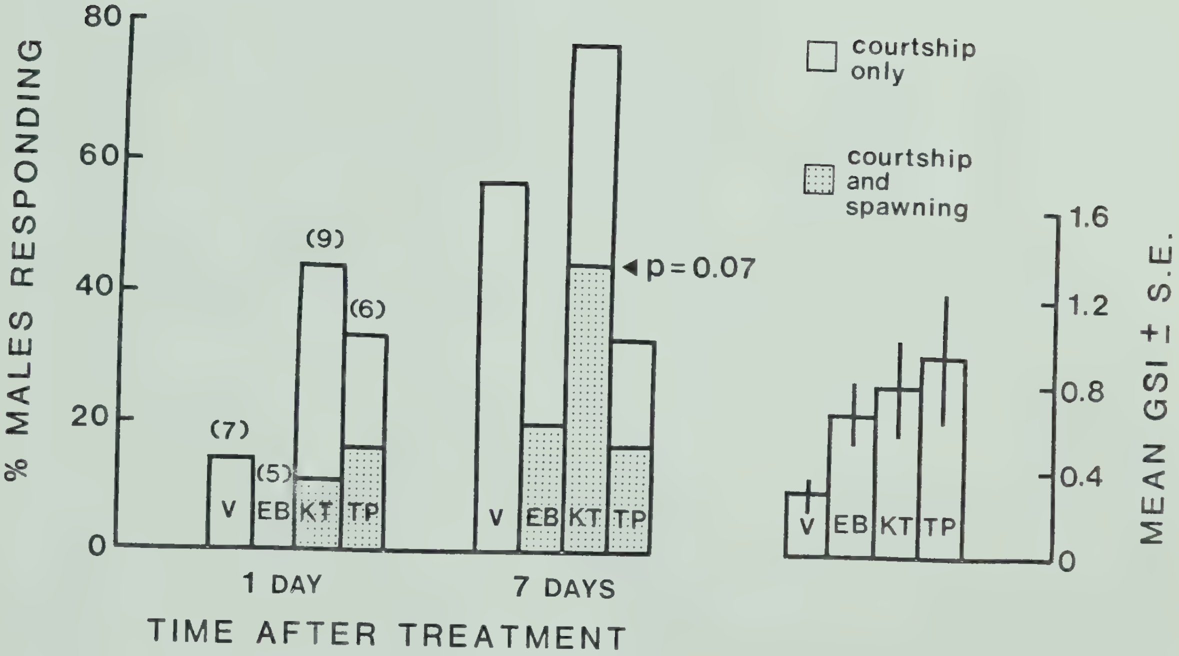


Fig. 4.2. Sexual behaviour of regressed male goldfish tested 1 and 7 days after a week of treatment with vehicle (V), estradiol benzoate (EB), 11-ketotestosterone (KT), or testosterone propionate (TP). (n) – sample size. p – calculated by Fisher's exact test, one-tailed, comparing each steroid treatment to the (V) group. GSIs are not different among groups, two-tailed ANOVA.



among treatment groups.

Exp. 4.3 - Brain Implants of Testosterone Pellets

Brain implants of T apparently increased the number of male goldfish courting females on the fourth test day, although this effect was not statistically significant (Fig. 4.3, $p=0.12$, Fisher's exact test, one-tailed), probably as a result of the small sample size. The control group became more sexually active on the subsequent tests, 10 and 20 days postimplantation, during which there were no differences between groups in the number of males either courting or spawning. Histological analysis of the brains was not performed. The GSIs were not different betweeen groups and were extremely low, with some animals having almost undetectable amounts of testicular tissue.

Exp. 4.4 - Brain Implants of Antisteroid Drugs

With the exception of the antiestrogen enclomiphene citrate, the antisteroid brain implants had no effect on male spawning behaviour, as more than 75% of the fish were sexually active in almost all treatment groups. However, Fig. 4.4 shows that four days after enclomiphene citrate implantation, the proportion of "30%" pellet-implanted males courting was smaller than that of the blank-implanted controls ($p<0.05$ for pellets in the NPO, $p<0.1$ for pellets in the Vs-pVv which becomes $p<0.05$ if the two Vs-pVv "blank" groups are combined, Fisher's exact test, one-tailed). For fish with "100%" pellets in the Vs-pVv, this depression persisted up to the eighth day postimplantation ($p<0.05$), while the "30%" animals showed complete behavioural recovery on this test day. The position of these pellets could not be verified histologically, presumably because they were completely absorbed by the surrounding tissues. GSIs were not different between groups.

D. DISCUSSION

Long term exposure to a high temperature (30°C), short photoperiod (8L:16D), and limited diet was used to produce sexually inactive male goldfish upon which the behavioural effects of steroid treatments could be examined. However, in the first three experiments, about 20% of the blank and vehicle-treated (control) males showed courtship behaviour on the first test day, even though they had been pretested for the lack of sexual activity. In exps. 4.2 and 4.3, the proportion of control males courting

Fig. 4.3. Sexual behaviour of regressed male goldfish tested at various times after implantation of either a beeswax (B) or testosterone (T) pellet into the supracommissural telencephalon. (n) – sample size. p – calculated by Fisher's exact test, one-tailed. GSIs are not different, two-tailed t-test.

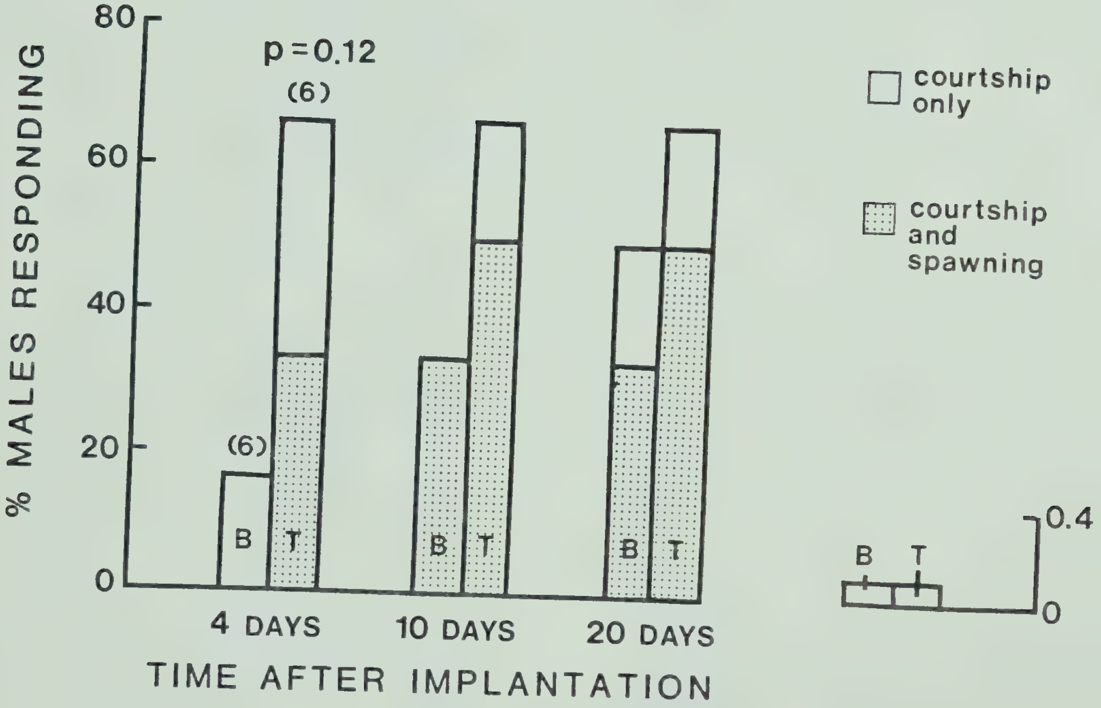
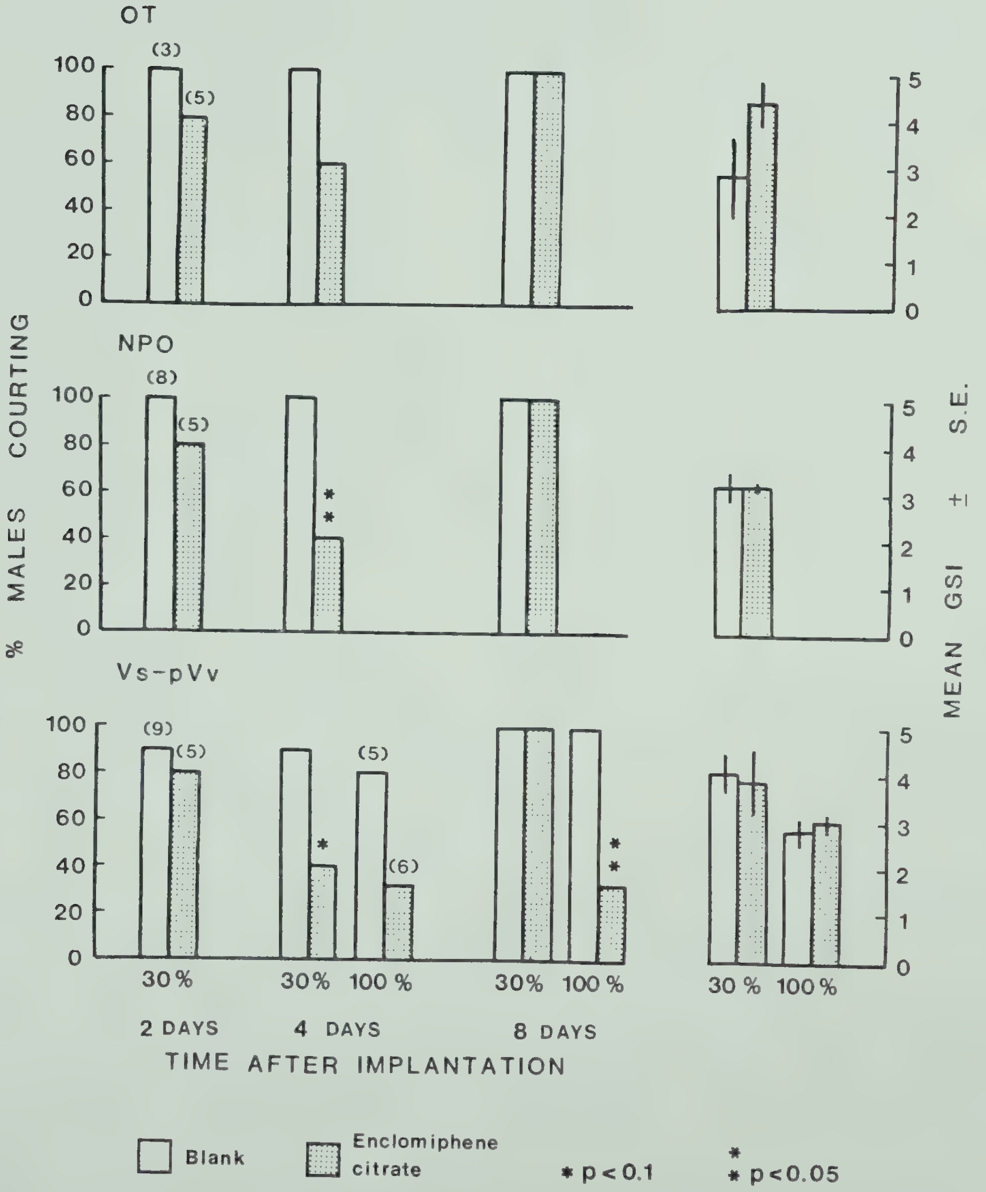


Fig. 4.4. Percentage of mature male goldfish courting when tested at various times after implantation of either a blank or enclomiphene citrate pellet into the optic tectum (OT), *nucleus preopticus* (NPO), or the supracommissural telencephalon (Vs–pVv). Pellets in the Vs–pVv contained either 30% or 100% by weight of the drug. (n) – sample size. p – calculated by Fisher's exact test, one-tailed. GSIs are not different, two-tailed t-test.



increased with each subsequent test, which suggests that experience and/or longer times spent at the experimental temperature of 20°C had a stimulatory effect and confounds the assessment of the steroid treatments. Perhaps the use of long term hypophysectomized males would provide more reliable controls.

The suggestion that androgen administration would increase the occurrence of male spawning behaviour was confirmed by exp. 4.1, where all regressed males implanted systemically with T pellets spawned on the fifth postimplant day. Encapsulation of some of the pellets, also observed by Hoar (1962), was noted at autopsy, which may have gradually reduced T release and resulted in the lowered amounts of spawning behaviour seen 40 days after implantation.

The i.p. injection of EB, 11KT, or TP into regressed male goldfish failed to increase the proportion of fish that either courted or spawned, although the results may suggest a stimulatory role for 11KT. The lack of significant treatment effects may have been due to the poor health of the animals in this experiment and consequent small sample sizes or to an inappropriate regimen of steroid administration. However, Wiegand and Peter (1980) were able to alter plasma lipid levels in goldfish with the same treatment protocol, indicating that such a regimen affects at least some physiological systems. In the present study, 11KT was more effective than either TP or EB in increasing the proportion of males spawning, although the effect was not significantly different from the controls (44% of 11KT-treated fish spawned, $p=0.07$, Fisher's exact test, one-tailed). 11KT is thought to be a major androgen in teleost fish (Ozon, 1972) and was more potent than T in inducing sex reversal or masculine secondary sexual characteristics in the medaka (*Oryzias latipes*) (Hishida and Kawamoto, 1970), the swordtail (*Xiphophorus helleri*) (Rastogi and Chieffi, 1975), and the guppy (*Poecilia reticulata*) (Takahashi, 1975). Other than a preliminary study that used intact male zebrafish (*Brachydanio rerio*) (Van den Hurk, 1977), the behavioural effects of this androgen have not been reported previously.

In both regressed male goldfish (exps. 4.1 and 4.2) and castrated male rats (Södersten et al., 1980), sustained-release T implants were more effective than T injections in facilitating male sexual behaviour. As well, the behavioural effectiveness of daily T injections in castrated male rats could be increased by as much as 50% if the

injections were synchronized with that time of the LD cycle in which pulses in androgen levels normally occurred (Södersten et al., 1980). This suggests that the target tissues have times of maximal sensitivity, and that sustained-release pellets are more likely to deliver adequate amounts of hormone at these times than are injections. A similar phenomenon also may exist in fish, as diel rhythms in 11KT and T have been described in the killifish (*Fundulus grandis*) (McGregor, 1981).

Experiment 4.3 tested techniques for manipulation of the steroid environment in an area of the brain previously shown to be important in male spawning behaviour (Chapts. II and III). Although implantation of T pellets into the supracommissural telencephalon did not result in statistically significant increases in the sexual behaviour of regressed males, the data at 4 days postimplantation (67% of T-implanted fish responding, $p=0.12$) are suggestive of such an effect. However, this experiment does not provide evidence for an exclusive behavioural site of T action in the supracommissural telencephalon; the results from implants into other brain areas are needed. Furthermore, assuming that the pellets released supraoptimal amounts of T, it appears that a systemic (100% males spawning, exp. 4.1) rather than a central (50% males spawning, exp. 4.3), route of administration was more effective. Several suggestions can be made to account for this difference. For example, spawning behaviour may depend upon the exposure of additional central nervous and/or peripheral tissues to androgens. As well, peripheral conversion of T into behaviourally efficient metabolites, possibly 11KT, may be required.

Factors other than the route of T administration also may have contributed to the higher levels of spawning activity seen in males with systemic T implants (exp. 4.1). The animals of exps. 4.2 (systemic injections) and 4.3 (T brain implants) were kept under the conditions designed to promote sexual regression for longer times than the animals in exp. 4.1, which, as well as resulting in smaller GSIs, might have altered factors that regulate sensitivity to androgen. In birds and mammals, photoperiod can alter behavioural sensitivity to hormone treatment, as T-treated castrates display less sexual behaviour under short days than long days (Campbell et al., 1978; McDonald and Liley, 1978). Although similar studies using castrated fish must be viewed with caution because of the problem of testicular regeneration, photoperiod effects on T sensitivity also may occur

(Wai and Hoar, 1963). In the ring dove (*Streptopelia risoria*), the effectiveness of T implants declines with the time following castration, suggesting that androgen sensitivity changes with hormonal status. Long-term castrates, which are relatively insensitive to T, show an increase in preoptic 5 beta-reductase, which converts T to behaviourally inactive metabolites (Steimer and Hutchison, 1981; Hutchison and Steimer, 1981).

Brain implants of antisteroid drugs (exp. 4.4) provided some evidence that steroids act centrally to stimulate male spawning behaviour in goldfish. Of the six anti-steroids used, only the antiestrogen enclomiphene citrate implanted into the NPO or Vs-pVv, but not the OT, significantly reduced the proportion of males courting. Both the NPO and Vs-pVv of the goldfish brain contain steroid-concentrating cells (Kim et al., 1978a) and are involved in reproductive behaviour (Demski and Hornby, 1982; Chapts. II and III). Whether the drug acted at these implant sites or at another site as a result of drug diffusion might be resolved by studies using implants into adjacent nuclei.

It would be premature to suggest a central role for estrogens based on the results of exp. 4.4. Although clomiphene citrate is effective in causing an increase in plasma GtH and ovulation in a number of teleosts, including goldfish (Peter, 1982), an exclusive antiestrogenic action has yet to be demonstrated in fish; possibly other types of steroid receptors interact with this drug. As well, the lack of effect of EB injections in increasing the proportion of spawning males argues against a role of estrogens in this regard.

It also would be premature to suggest that the lack of effect of antiandrogens negates a role for androgenic hormones. Using the example of an antiandrogen that has been used in fish, cyproterone acetate (CA), the following studies suggest that the timing, duration, and ratio of drug:endogenous T used in this experiment may not have been suitable:

1. CA, when given simultaneously with the androgen, failed to block the masculinizing effects of TP or 11KT on female swordtails (Rastogi and Chieffi, 1975); however, when administered 3 h prior to androgen treatment, CA effectively reduced the concentration of ^{3}H-T in the plasma, testes, and liver of juvenille rainbow trout (*Salmo gairdneri*) (Schreck, 1973). Interestingly, no effect of CA was seen on ^{3}H-T uptake in the kidney, spleen, gall bladder, brain, or epaxial muscle, suggesting tissue specificity of CA action in

the trout.

2. When administering CA to adult males, several weeks of treatment appear to be necessary. In the stickleback (*Gasterosteus aculeatus*), two weeks of CA treatment were needed before ultrastructural changes in the androgen-dependent cells of the kidney were seen (Mourier, 1976), and three weeks of CA treatment reduced aggressive and courtship behaviour in male sticklebacks (Rouse et al., 1977). As the 30% enclomiphene citrate pellets lost their depressive effect on behaviour 8 days after implantation, 30% antiandrogen pellets may not have contained enough drug to be effective; it is not known how long the 100% pellets would have delivered sufficient amounts of drug.

3. CA-induced reduction of nuptial colouration and delayed onset of nest building were observed in winter-caught, but not spring-caught, sticklebacks (Rouse et al., 1977). As the winter animals were thought to have had lower pretreatment androgen levels, CA may only be effective at high drug:androgen ratios. Along these lines, in mammals, it is generally accepted that more androgen is needed to restore behaviour in sexually inactive castrates than to maintain behaviour in males whose replacement therapy begins immediately after castration. Hence, the antiandrogen, flutamide, blocked T-induced initiation, but not T-induced maintenance, of sexual behaviour in castrated male rats (Gladue and Clemens, 1980).

4. Antisteroid administration to intact animals may cause compensatory regulatory responses. If the drug diffused into the ventrobasal hypothalamus, then one might predict that an increase in GtH and androgens might occur due to blockage of the negative feedback effects of endogenous androgens (Peter, 1982), thus neutralizing the effect of the treatment.

In summary, systemic T implants were very effective in restoring spawning activity in sexually regressed male goldfish. The results of injection experiments were less satisfactory, perhaps because of the route of steroid administration or the highly regressed condition of the fish in this experiment; nevertheless, 11KT appeared to be more effective than either EB or TP. Brain implants of T weakly stimulated, while the antisteroid drug enclomiphene citrate decreased spawning behaviour when implanted into the NPO or Vs-pVv. The results suggest a central site of action of androgens in facilitating male spawning behaviour in the goldfish.

V. EFFECTS OF SEXUAL STIMULI ON GONADOTROPIN AND MILT LEVELS IN THE GOLDFISH

A. INTRODUCTION

In many male mammals and birds, exposure to sexual stimuli results in acute elevations of circulating luteinizing hormone (LH) and testosterone (T). These hormonal changes can be induced by the sight and/or smell of a female, and thus probably occur prior to actual contact and mating in the normal situation (Hutchison, 1978; Harding, 1981; O'Connell et al., 1981). In fact, anticipatory rises in these hormones or their dependent physiological processes can be evoked even in the absence of a female if the male is presented with a situation where sexual activity has previously occurred (Anon., 1970; Kamel et al., 1975; Graham and Desjardins, 1980). Except for a preliminary report of the present work (Kyle et al., 1979), similar observations have not been published previously for poikilothermic vertebrates, although indirect evidence indicates that sexual stimuli, when presented chronically, can activate the pituitary-gonadal axis of teleost fish. For example, several weeks of exposure to the opposite sex facilitated endocrine-controlled events such as maturation of eggs and ovulation in a variety of teleost species (Aronson, 1945; 1965; Chien, 1973; Eaton and Farley, 1974; Chen and Martinich, 1975) and the development of the nuptial colouration in male sticklebacks (*Gasterosteus aculeatus*) (Reisman, 1968). In addition, stimulation of the male endocrine system has been suggested as a mechanism by which exposure to a female or her holding water caused the onset of nest building in several anabantids and cichlids, (Liley, 1982).

In the present work, we first studied the effect of short-term exposure to sexual stimuli on two indexes of the reproductive status of the male goldfish (*Carassius auratus*): the concentration of serum "Con A II" gonadotropin (GtH) and the volume of milt that could be expressed by hand-stripping. "Con A II" or "maturational" GtH, one of two gonadotropins reported in teleost fish, was of interest because its "LH-like" involvement in steroidogenesis and spermiation (Idler and Ng, 1979) suggested that, as with mammalian LH, circulating levels may be altered by social conditions. As well, GtH was substantially elevated in spawning male carp (*Cyprinus carpio*) (Fish Reproductive

Physiology Group, 1978) and white suckers (*Catostomus commersoni*) (N.E. Stacey, D.S. MacKenzie, and A.L. Kyle, unpublished results) as compared to mature, but prespawning individuals. The volume of expressible milt was measured not only because its dose-dependent relationship to exogenous GtH in hypophysectomized goldfish (Yamazaki and Donaldson, 1968) suggested that it would be a good indicator of endogenous GtH changes, but also because of the observation that apparently nonspermiating males often had expressible milt after sexual activity. The second and third portions of this work attempted to determine whether the observed changes in GtH concentrations and milt volumes were causally connected.

B. METHODS

Animals and Maintenance

Goldfish were obtained from the Grassyforks Fisheries Co., Martinsville, Indiana and held in 3600 or 1000 litre flowing-water tanks at 14°C, under a simulated natural spring and summer photoperiod (Edmonton), for various times prior to each experiment. Animals were fed twice daily with commercial fish food (Ewos pellets). Only mature males, which had tubercles on the pectoral fins and operculum and expressible milt were used; body weights ranged from 15-30 g.

Preparation of Stimulus Fish

Either a receptive female or pairs of spawning goldfish provided the sexual stimuli for the experimental males; the use of the latter ensured that the test males were at least exposed to a spawning stimulus, irrespective of their own behavioural response (several males may court and spawn with a single female). In both situations, normal female reproductive behaviour was induced by the intramuscular injection of prostaglandin F_2alpha (PG) at 200 ng/g body weight (Stacey, 1976; Stacey and Peter, 1979). The "stud" males of the stimulus spawning pairs were fish that had previously demonstrated persistent and vigorous courtship of PG-injected females. All experimental aquaria contained a spawning substrate of artificial, floating plants and were screened as much as possible from any outside disturbances.

Milt and Blood Samples

Fish were anesthetized in solutions of either 0.1% tricaine methanesulfonate or 0.05% 2-phenoxyethanol prior to milt or blood samples. Before stripping the fish of milt, urine was first expelled by applying a light pressure around the urogenital pore and blotting the fluid with tissue paper. Milt expressed during subsequent gentle stroking of the abdomen, in an anterior to posterior direction, was immediately aspirated into a 20 or 50 *ul* glass micropipette. The use of excessive force to strip milt was avoided, as this ruptured the blood vessels around the urogenital ducts and did not noticeably increase the volume of milt collected. The micropipettes were spun for 15 min in a hematocrit centrifuge and the volumes of milt and packed sperm were measured for calculation of the % seminal plasma content. Care was taken to centrifuge the milt promptly after sampling, as the supernatant and packed cells did not separate cleanly if the samples were left refrigerated overnight.

When required, blood was taken from the caudal vasculature while the fish were anesthetized for milt stripping. For experiments that ran over several days, blood sampling was done within an hour of the same time each day to minimize the effects of possible daily GtH fluctuations (Hontela and Peter, 1978). The blood was kept on ice until clotted, centrifuged at 5°C, and the serum stored at −28°C. Serum GtH levels were later determined by radioimmunoassay (Crim et al., 1976; Hontela and Peter, 1978, 1980).

Study 5.1 - Effects of Sexual Stimuli on GtH and Milt Levels

The first experiment in this series examined GtH levels in male goldfish after exposure to either another male or a receptive female. It was observed that some of the female-exposed males appeared to have more expressible milt at the end of this experiment than at the beginning, and the results of a preliminary study (N.E. Stacey and R. Billard, unpublished results) agreed with this observation. The second and third experiments, therefore, tested the hypothesis that males exposed to sexual stimuli, in the form of spawning pairs of goldfish, would show increases in both GtH and expressible milt levels on a short-term basis. To characterize the sensory information required to mediate these physiological changes, the fourth and fifth experiments manipulated the type of sensory cues available to the test male. The fifth experiment also questioned whether exposure to a homosexual spawning pair would have the same effects as

exposure to a heterosexual pair of goldfish.

Exp. 5.1.1 - GtH Levels in Males Exposed to Other Males or Females

A small group of males from a May stock of goldfish were warmed to 20°C for one week and then individually placed in 65 litre glass aquaria, at 20°C. The following morning, either a male or a PG-injected female stimulus fish was added to each aquarium. Experimental males were removed and bled for GtH assay either immediately upon addition of the stimulus fish (0 min) or 20, 40, or 60 min later; this protocol was repeated over several days.

Exp. 5.1.2 - Milt and GtH Levels in Males Exposed to Spawning Goldfish

This experiment was performed in July, using a stock of fish obtained several months earlier. Seventeen mature males were initially (0 h) stripped of milt and placed into three 225 litre tanks, at 14°C. Ten of these males (stimulated) were divided equally between two tanks that each contained two pairs of actively spawning goldfish, while the remainder (controls) were added to the third, empty tank. The males were repeatedly removed, stripped of milt, and returned to the experimental tanks after 1, 3, 6, 12, and 24 h; blood samples were taken at 1 and 3 h. This protocol was repeated on the following day with a new group of goldfish.

Exp. 5.1.3 - Milt and GtH Levels in Males Exposed to Spawning Goldfish

This experiment modified the protocol of exp. 5.1.2. Experimental males were moved into the test tanks, at 14°C, on the afternoon preceeding the test day. On the test day, a presample of blood and milt was taken, and stimulus pairs of spawning goldfish were added to two of three tanks one hour later (0 h). Blood and milt samples were taken at 2 h and milt volumes were determined again at 6 and 24 h. This experiment was performed in August, using a stock of fish obtained several months earlier.

Exp. 5.1.4 - Milt and GtH Levels in Males Visually Exposed to Spawning Goldfish

Male goldfish, were stripped of milt and given one of the following three treatments:

1. Isolated - males were placed singly in a 65 litre glass aquarium, at 20°C, that was covered on all sides by black plastic.
2. Contact - males were placed individually with a stimulus pair of spawning goldfish.
3. Visual - males were isolated as in treatment 1, except that the long edge of the

aquarium was uncovered and butted against the "Contact" aquarium such that the isolated animal could observe the fish spawning in the adjacent tank.

Two hours later, the experimental males were stripped of milt, bled, and killed for determination of their gonadosomatic index (GSI=gonad weight x 100/total body weight). This experiment was performed in March.

Exp. 5.1.5 - Milt and GtH Levels in Males in Contact with or Separated from Spawning Goldfish

Sixty-five litre glass aquaria, at 20°C, were covered on all sides by black plastic and divided crosswise by loosely fitting, clear plexiglass partitions, each perforated by 6 holes 1.5 cm in diameter. Water was circulated between the tank halves by an air lift system, made by passing air tubing within a larger glass sleeve through the partition. Stimulus fish were first arranged in the test tanks and allowed to spawn; the experimental males were then stripped of milt and individually added to one of five treatment tanks (0 h):

1. Isolated - the test male was placed on one side of the partition and the other side was left empty.
2. Male/female-contact - the test male was placed with a heterosexual spawning pair on one side of the partition.
3. Male/female-separated - the test male was placed on the other side of the partition in the aquarium described above.
4. Male/male-contact - the test male was placed with a homosexual pair of spawning goldfish on one side of the partition. The male performing the female role was previously olfactory tract-sectioned, an operation that reduces male behaviour (N.E. Stacey and A.L. Kyle, unpublished results), and injected with PG on the test day, which induces female behaviour in males as well as in females (Stacey, 1981).
5. Male/male-separated - the test male was placed on the other side of the partition in the aquarium described above.

Blood and milt samples were taken at 2 h and the animals were killed for GSI determinations. This experiment was performed in April.

Study 5.2 - Hormonal Effects on Milt Volumes

Study 5.1 showed that exposure to sexual stimuli caused a rapid increase in milt volumes and serum GtH concentrations. As gonadotropin reinstates spermiation in hypophysectomized goldfish (Yamazaki and Donaldson, 1968), it is possible that the sexually stimulated increase in milt is mediated by GtH. The following series of experiments examined whether injections of various gonadotropins or other hormones could evoke changes in milt volume similar to those seen in response to a spawning situation.

Exp. 5.2.1 - Carp GtH and hCG

Male goldfish were stripped of milt (0 h), and injected intraperitoneally (i.p.) with one of: teleost saline (5 *u*l/g body weight), carp GtH (cGtH; 25 ng/g body weight; gift from B. Breton), or human chorionic gonadotropin (hCG; 2.5 I.U./g body weight; Sigma). Each treatment group was kept separately in 225 litre flowing-water tanks, at 14°C, and repeatedly stripped of milt 1, 3, 6, 9, 12, and 24 h after injection. This experiment was done in July with fish that had been obtained several months earlier.

Exp. 5.2.2 - Carp GtH and Goldfish Pituitary Extract

Male goldfish were stripped of milt and injected i.p. with one of: teleost saline (5 *u*l/g body weight), cGtH (25 ng/g body weight), or freshly dissected goldfish pituitaries homogenized in teleost saline (65.4 *u*g wet weight tissue/g body weight, which was slightly less than 2 pituitaries/fish). Treatment groups were kept separately in 225 litre tanks, at 14°C, and repeatedly stripped of milt 1, 3, 6, 9, 12, and 24 h after injection.

Exp. 5.2.3 - Adrenalin and Goldfish Urophyseal Extract

Two preliminary experiments were conducted, at 14 °C. In the first, males were injected with teleost saline (2 *u*l/g body weight) or adrenalin (40 *u*g/g body weight; Sigma) and stripped of milt 1 h later. In the second experiment, the urophysis and surrounding tissue was dissected from 15 goldfish, lyophilized, and reconstituted in saline (210 mg/4 ml). Males were injected with 200 *u*l of saline or urophyseal homogenate (about 20 mg dry weight tissue/fish) and stripped of milt 1 h later.

Study 5.3 - Effect of Sexual Stimuli or Gonadotropin on Testicular Hydration

Gonadotropin-induced spermiation in fish has been described as "the release of spermatozoa into the sperm duct by thinning of the semen" (Yamazaki and Donaldson, 1968) and is concomitant with gonadal hydration (Clemens and Grant, 1964, 1965). This

study measured the changes in testicular water content induced by sexual stimuli (exp. 5.3.1) or injection of hCG (exp. 5.3.2).

Exp. 5.3.1 - Sexual Stimuli and Testicular Hydration

Thirty male goldfish obtained in March were divided equally between three 225 litre flowing-water tanks, at 14°C. The next morning, males were stripped of milt (0 h) and spawning pairs of goldfish were added to two of the three tanks. All males were stripped of milt at 2 h and one tank of stimulated males were stripped again at 4 h. At this time, all animals were killed; the testes were removed, weighed, and left to dry for 10 days at 60°C. The reduction in weight after drying was taken as an estimate of testicular water content.

Exp. 5.3.2 - hCG and Testicular Hydration

Males from a May stock of goldfish were held at 14°C and injected with either teleost saline (5 *ul*/g body weight) or hCG (2.5 I.U./g body weight) and killed 12 h later. The percentage of testicular water was determined as described in exp. 5.3.1.

C. RESULTS

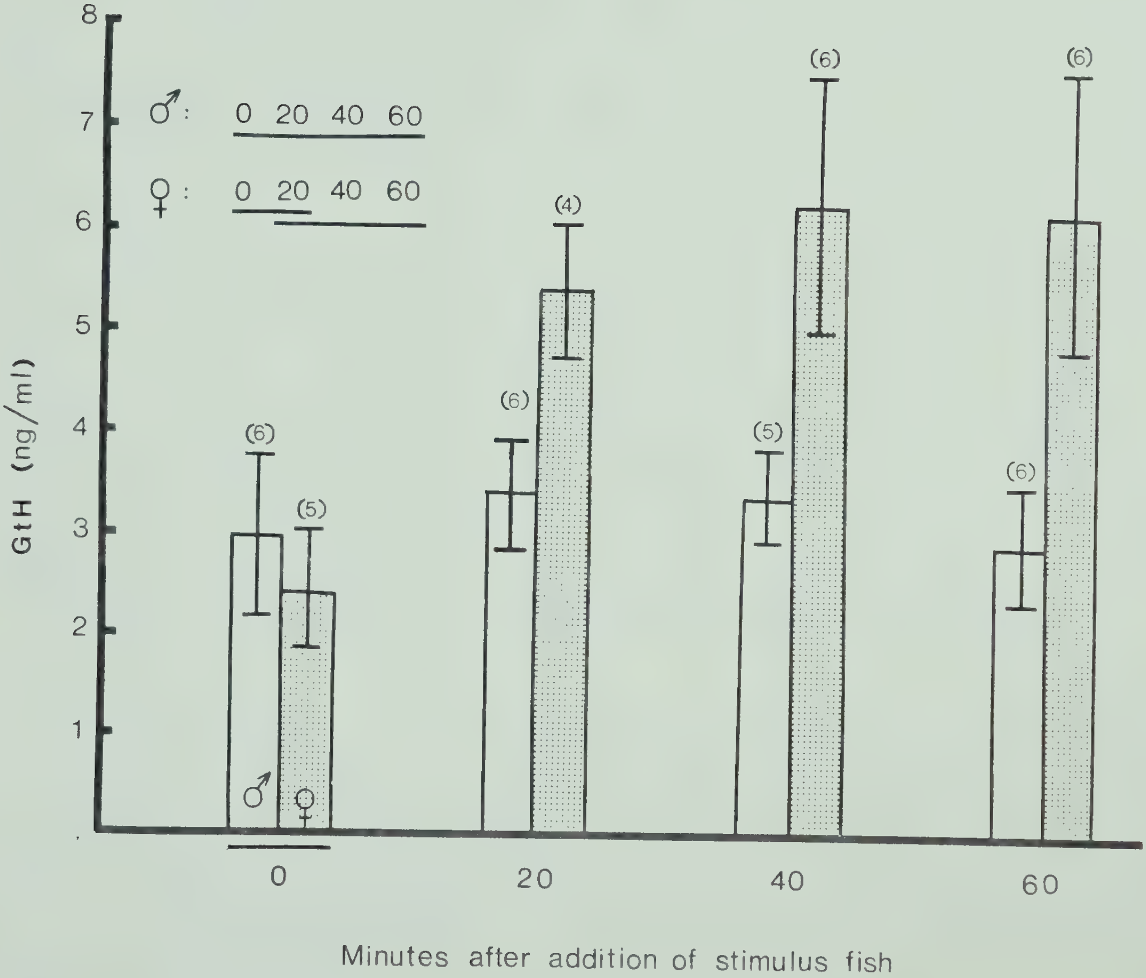
Study 5.1 - Effects of Sexual Stimuli on GtH and Milt Levels

Gonadotropin Levels

Fig. 5.1.1 shows that 40 or 60 min of exposure to sexually receptive females significantly increased serum GtH concentrations in male goldfish ($p<0.05$, ANOVA and Duncan's multiple range test) over the initial (0 min) values. In contrast, the GtH levels of those males exposed to other males did not change over the duration of the test. The GtH values for the female-exposed males also were higher ($p<0.05$, two-tailed t-test) than those for the male-exposed males at the 20, 40, and 60 min sample times.

Similar elevations in GtH levels are shown in Fig. 5.1.2; the serum GtH concentrations of the sexually stimulated males were almost double ($p<0.05$, one-tailed t-test) those of the unstimulated controls at 1 h, but not significantly different at 3 h ($p<0.1$). Exp. 5.1.3 employed a different protocol than the previous experiment with regard to the timing of blood and milt samples and demonstrated a different pattern of GtH change. GtH values for the unstimulated controls dropped significantly (Fig. 5.1.3, $p<0.01$, Wilcoxon matched-pairs signed-ranks test, two-tailed) between the presample

Fig. 5.1.1. Mean GtH concentrations (± S.E.) in male goldfish held at 20°C and exposed to either another male or a receptive female. Those treatments underlined by the same bar are not significantly different at $p<0.05$ (two-tailed t-test – treatments; ANOVA and Duncan's multiple range test – times). (n) – sample size.



and 2 h sample time, while there was no such decline in the stimulated group. Thus, the GtH samples were significantly higher ($p<0.05$, one-tailed t-test) in the stimulated males than the nonstimulated males at 2 h because of the smaller decrease following blood sampling.

The GtH concentrations shown in Fig. 5.1.5 were extremely variable, with 100 fold differences occurring within the same treatment group. As well, the GtH concentrations of some samples could not be calculated, as they exceeded the upper limit of the standard curve (>100ng/ml) and were, therefore, assigned the value of 100 ng/ml. A nonparametric ANOVA (Kruskal-Wallis test) revealed no differences between groups. However, pairwise comparisons (Mann-Whitney U test, one-tailed) found that the GtH levels of the two "contact" groups (male/female-contact and male/male-contact) were significantly higher than the isolates and not different from each other, while the two "separated" groups (male/female-separated and male/male-separated) were neither different from each other nor from the isolated fish. Accordingly, the data were combined into three, instead of five, treatments: isolated, contact, and separated, and the appropriate statistics were recalculated. Significant differences now were found between groups, with the contact group having higher levels of GtH than either the isolated or separated fish (Fig. 5.1.5). Exp. 5.1.4 failed to find any changes in GtH as a result of either visual exposure to or contact with a spawning pair of goldfish.

Milt Levels

Figs. 5.1.2 and 5.1.3 show that significantly more milt ($p<0.05$, one-tailed t-test) could be stripped from males after 1 to 24 h of exposure to pairs of spawning goldfish than from males kept in all-male groups. Although the behaviour of individuals in the group could not be determined easily, most males were observed to court the females at some time during the test.

If contact between test males and a spawning pair was prevented, either by housing the male in an adjacent tank where the spawning fish could be only seen (exp. 5.1.4) or by placing the male in the same tank but separated from the spawning fish by a perforated partition (exp. 5.1.5), then the milt volumes after 2 h of treatment were not different from those of isolated males (Figs. 5.1.4 and 5.1.5). These test males appeared to be aware of the adjacent, but inaccessible, spawning activity and spent much of the

test time oriented towards or swimming along the side of the tank or partition adjacent to the spawning fish. However, if contact was allowed between the test males and a spawning pair, whether that pair was heterosexual (exp. 5.1.4 and 5.1.5) or homosexual (where one male performed the female role, exp. 5.1.5), then milt levels were significantly elevated over those of the isolated controls (Figs. 5.1.4 and 5.1.5, $p<0.05$, ANOVA and Duncan's multiple range test). GSIs were not different among the treatment groups of exps. 5.1.4 or 5.1.5.

In all of the experiments in this study, the % seminal plasma values were not different between treatments at any sample time and averaged about 70%.

Sexually Active vs. Inactive Males

The first four experiments in this series provided no opportunity to correlate milt volumes or GtH levels with the behaviour of individual fish, either because almost all males exposed to a female or spawning pair were sexually active (exps. 5.1.1 and 5.1.4) or because the males were tested in large groups over several hours and, therefore, difficult to observe individually (exps. 5.1.2 and 5.1.3). In exp. 5.1.5, however, 5 of the 13 males placed in contact with the heterosexual spawning pair and 5 of the 12 males placed with the homosexual spawning pair did not court or spawn, when periodically observed during the 2 h test. The data for the combined "contact" group, therefore, were divided into sexually active and inactive categories and compared with the isolated controls (Fig. 5.1.6). After 2 h of exposure to a spawning pair, sexually active males had higher milt and GtH levels than either the inactive or isolated fish ($p<0.05$, ANOVA and Duncan's multiple range test – milt, Kruskal-Wallis test – GtH). In contrast, sexually inactive males were not different from isolated fish in either the volume of expressible milt or concentration of GtH. The initial (0 h) milt volumes and GSIs were similar among groups (ANOVA and Duncan's multiple range test).

Study 5.2 - Hormonal Effects on Milt Volumes

In the first experiment (5.2.1), injection of gonadotropin, either carp GtH or hCG, did not significantly increase milt volumes over those of the saline controls until 6 h post-injection (Fig. 5.2.1; ANOVA and Duncan's multiple range test). The milt volumes of the two treatment groups continued to increase over subsequent samplings. In the second experiment, milt volumes from both the cGtH and pituitary homogenate-injected

Fig. 5.1.2. Milt volumes and GtH concentrations (mean ± S.E.) of male goldfish held at 14°C and repeatedly sampled at various times while kept either in an all-male group (controls – C) or with pairs of spawning goldfish (stimulated – S). p – calculated by one-tailed t-test. (n) – sample size.

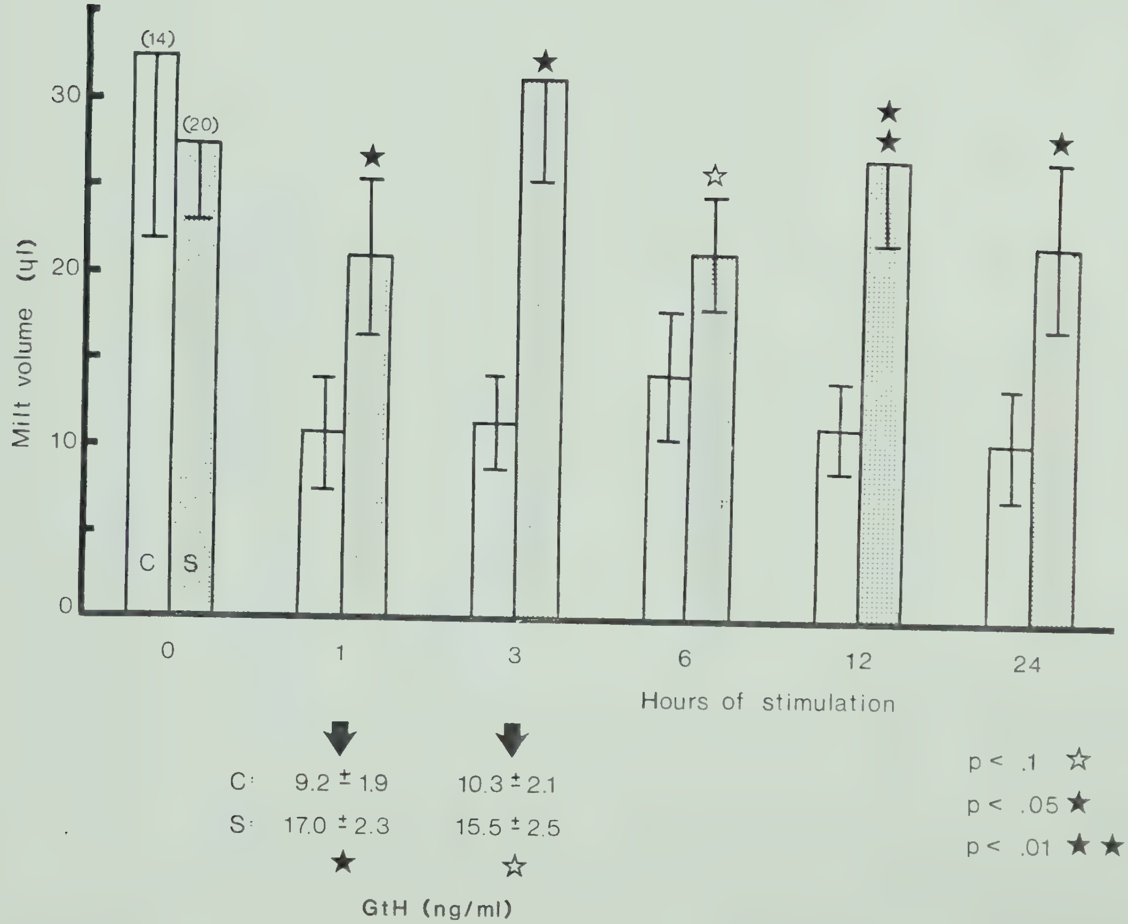


Fig. 5.1.3. Milt volumes and GtH concentrations (mean ± S.E.) of male goldfish held at 14 °C and repeatedly sampled at various times while kept either in an all-male group (controls – C) or with pairs of spawning goldfish (stimulated – S). p – calculated by one-tailed t-test (milt and GtH, C vs. S) or Wilcoxon matched-pairs signed-ranks test, two-tailed (GtH, presample vs. 2 h). (n) – sample size.

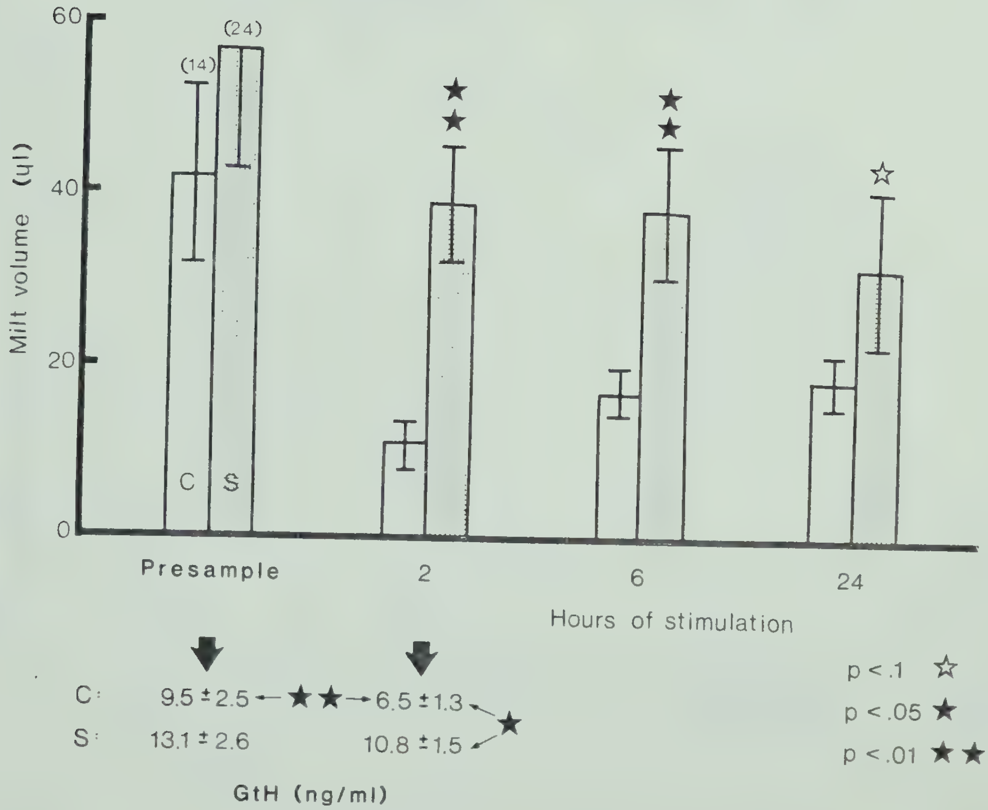


Fig. 5.1.4. Milt volumes, GtH concentrations after 2 h of stimulation, and GSIs (mean ± S.E.) of male goldfish that were held at 20°C and isolated from (I), in contact with (C), or visually exposed to (V) a spawning pair. p – calculated by ANOVA and Duncan's multiple range test, only significant values shown. (n) – sample size.

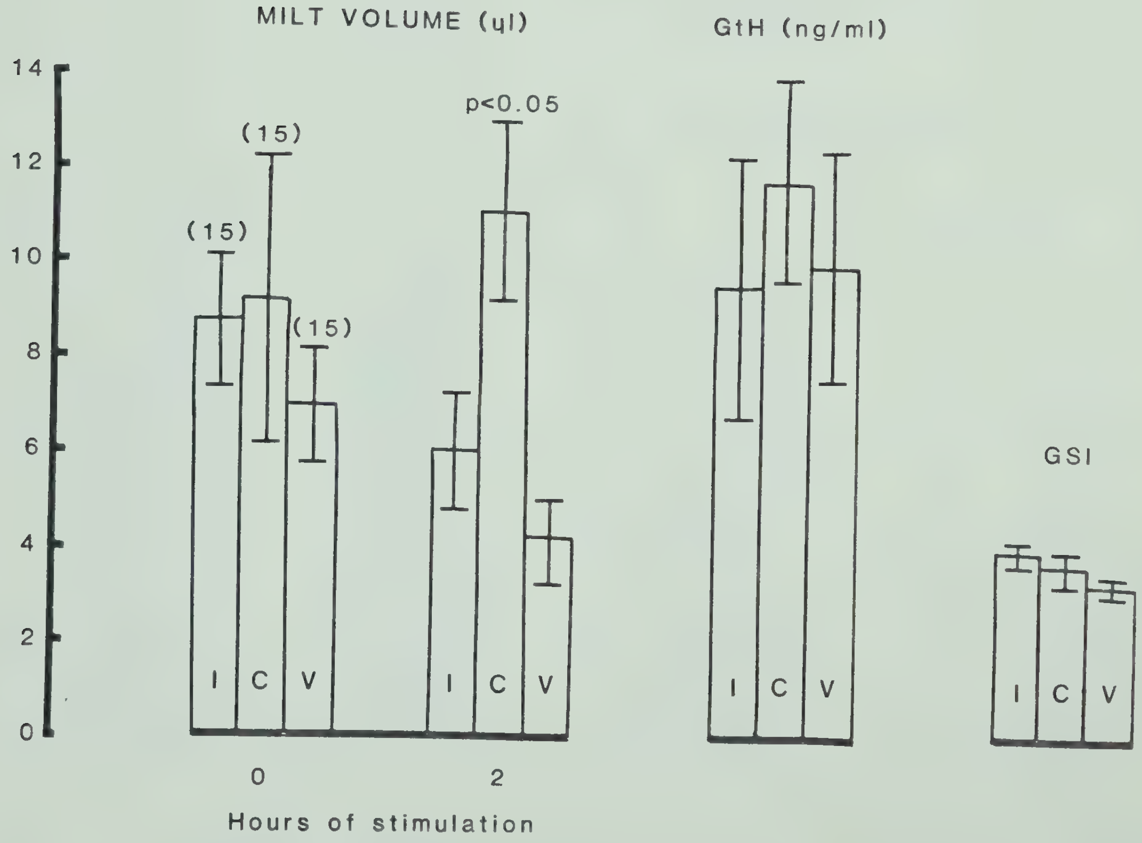


Fig. 5.1.5. Milt volumes, GtH concentrations after 2 h of stimulation, and GSIs (mean ± S.E.) of male goldfish that were held at 20°C and isolated from (I), in contact with (C), or separated with a perforated partition from (S) either a heterosexual (♀) or homosexual (♂) pair of spawning fish. p – calculated by ANOVA and Duncan's multiple range test (milt and GSI) or Kruskal–Wallis test (GtH; data combined to yield three groups: I, C, and S), only significant values shown. (n) – sample size.

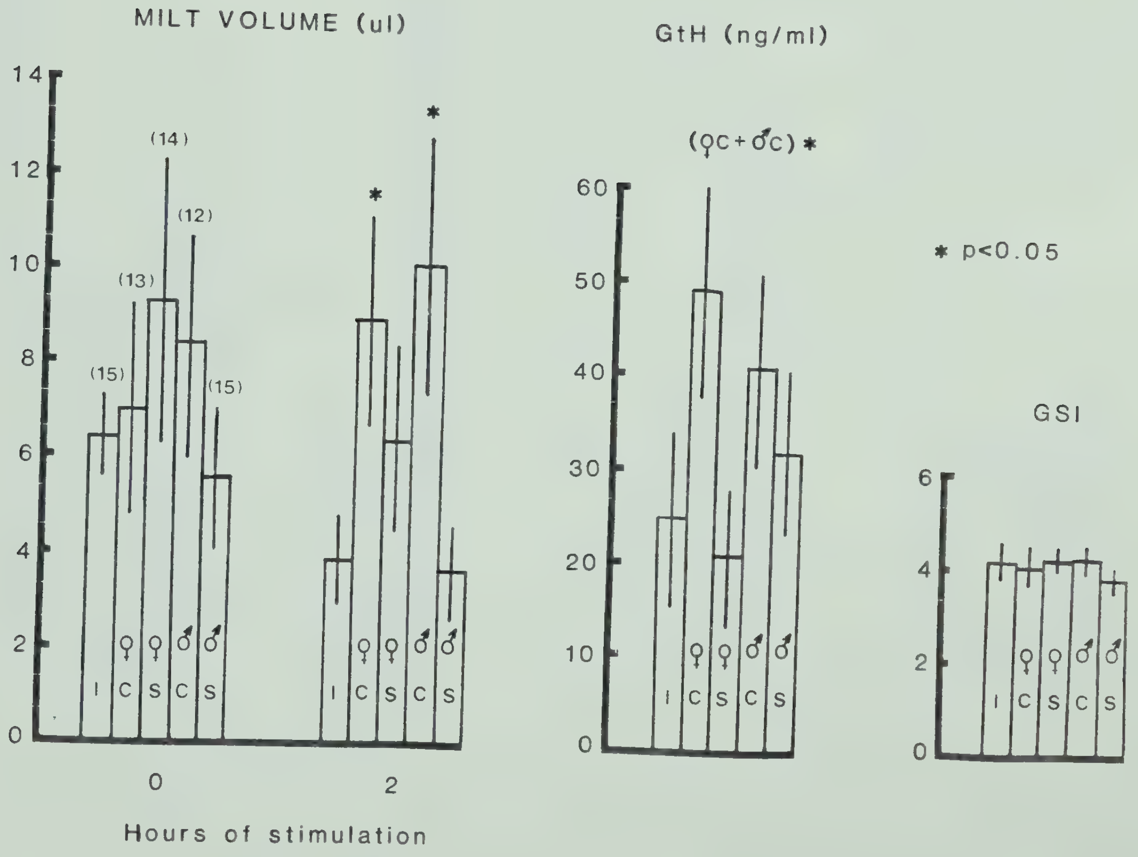
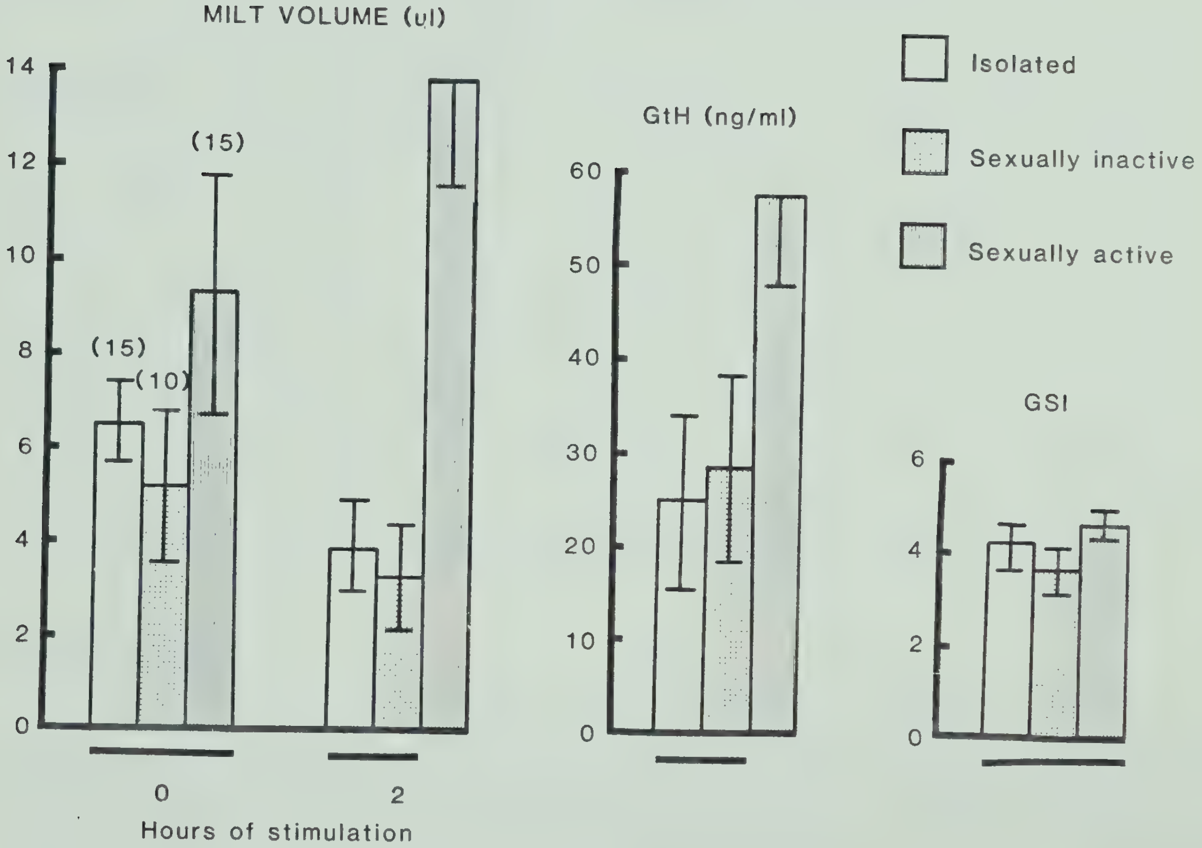


Fig. 5.1.6. Milt volumes, GtH concentrations after 2 h of stimulation, and GSIs (mean ± S.E.) of isolated, sexually inactive, and sexually active male goldfish held at 20°C. Those values underlined by the same bar are not significantly different at $p<0.05$ (ANOVA and Duncan's multiple range test – milt and GSI, Kruskal–Wallis test – GtH). (n) – sample size.



groups were significantly higher at 3 h, but not at 1 h, post-injection (Fig. 5.2.2; ANOVA and Duncan's multiple range test). Again, milt volumes of hormone-treated males continued to increase over subsequent sampling times, and the three treatment groups were all significantly different from each other at 24 h. In both experiments, the % seminal plasma values for the treatment groups were highly variable and not different from each other at any sample time. Injection of adrenalin or urophyseal homogenate had no effect on milt volume.

Study 5.3 - Effect of Sexual Stimuli or Gonadotropin on Testicular Hydration

In exp. 5.3.1, no difference ($p=0.43$, two-tailed t-test) was found between the mean testicular water content of sexually stimulated males that had been stripped of milt 2 h before gonad removal (77.30 ± 1.51 S.E.) and those stripped immediately before sacrifice (78.31 ± 0.43 S.E.); therefore, the % water values of these groups were combined. Table 5.1 shows that exposure to spawning pairs of goldfish increased milt volumes over those of the unstimulated controls, but that this was not paralleled by an increase in testicular water. In contrast, the testicular water content of hCG-injected fish was significantly elevated over saline controls (Table 5.2, $p<0.05$, Mann-Whitney U test, two-tailed) at a time when milt levels would also be elevated (12 h post-injection; Fig. 5.2.1). The GSIs were not different between treatment groups in both experiments.

D. DISCUSSION

This is the first report in fish of rapid elevations in either GtH concentrations or expressible milt volumes after exposure to sexual stimuli (either a receptive female or a pair of spawning goldfish). At 20°C, serum GtH concentrations were increased at 20 min and remained elevated for up to 2 h as compared to nonstimulated controls (exps. 5.1.1 and 5.1.5). A similar time course occurred at 14°C; GtH levels of sexually stimulated males were elevated at 1 h (the first sample time in exp. 5.1.2) and 2 h (exp. 5.1.3) and were no longer different from the control fish at 3 h (exp. 5.1.2). The transitory nature of the gonadotropin increase in response to sexual stimuli also was noted for LH in mammals, with concentrations peaking in about 15 min and declining to baseline levels in about 60 min (Maruniak and Bronson, 1976; Kamel et al., 1977; Coquelin and Bronson, 1979).

Fig. 5.2.1. Milt volumes (mean ± S.E.) of male goldfish held at 14°C and sampled at various times after injection of saline, cGtH, or hCG. Those treatments underlined by the same bar are not significantly different at $p<0.05$ (ANOVA and Duncan's multiple range test). (n) – sample size.

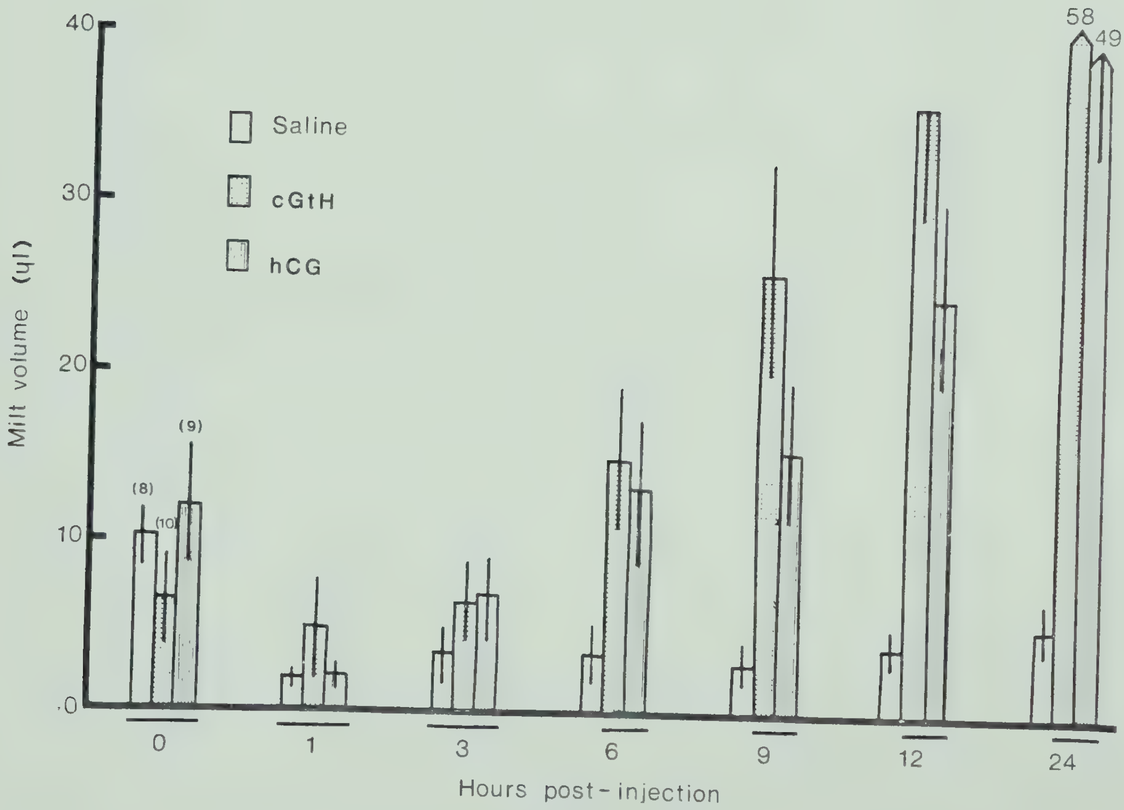


Fig. 5.2.2. Milt volumes (mean ± S.E.) of male goldfish held at 14°C and sampled at various times after injection of saline, cGtH, or goldfish pituitary homogenate. Those treatments underlined by the same bar are not significantly different at $p<0.05$ (ANOVA and Duncan's multiple range test). (n) – sample size.

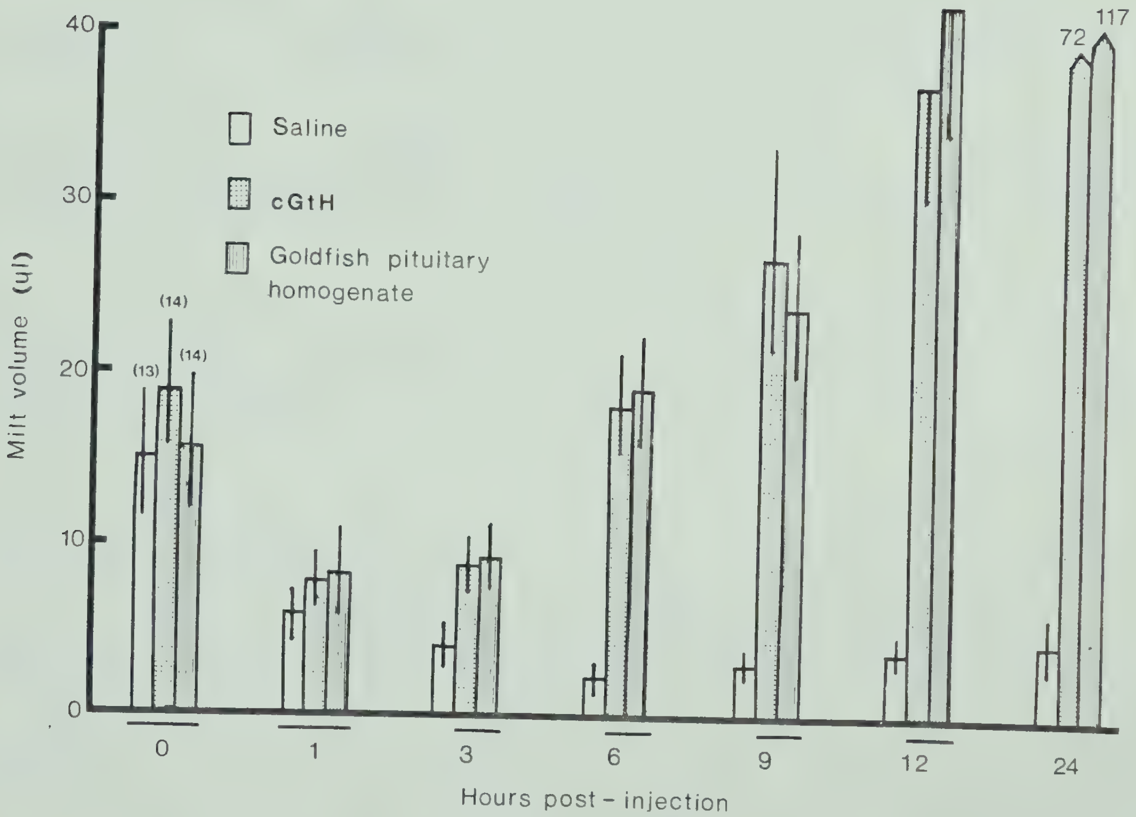


Table 5.1. Milt volumes and testicular water content of sexually stimulated male goldfish held at 14°C and sacrificed after 4 h of stimulation. See Table 5.2 for symbol legend.

	CONTROL	STIMULATED	P ▽
Sample size	10	20	
% water ◇	77.6±1.5	77.8±0.7	n.s.
GSI ◇	4.92±0.38	4.56±0.32	n.s.
μl milt - 0 h ◇	6.54±1.40	9.54±1.72	n.s.
μl milt - 2 h ◇	2.93±0.66	8.73±1.57	$p<0.01$

Table 5.2. Testicular water content of hCG-injected male goldfish held at 14°C and sacrificed 12 h postinjection.

	SALINE	hCG	P ▽
Sample size	5	5	
% water ◆	74.3 (52.1-76.6)	80.5 (75.8-81.9)	$p<0.05$ ▼
GSI ◇	3.91±0.89	3.40±0.85	n.s.

◇ mean±S.E.

◆ median (range)

▽ t-test, 2-tailed, except where noted

▼ Mann-Whitney U test, 2-tailed

In exp. 5.1.3, the GtH concentrations of control fish declined between serial blood samples, a phenomenon also noted by others for goldfish (Chang et al., 1982) and mammals (Harding, 1981). This decline did not occur to the same extent in males exposed to a spawning pair, suggesting a facilitation of GtH secretion at this time (2 h at 14°C).

Exps. 5.1.4 and 5.1.5 were performed one week apart at 20°C and used males with similar initial milt levels and GSIs. Even so, elevated GtH concentrations due to contact with a spawning pair were found only in the latter experiment. However, the fish in the former experiment were more sexually active and may have begun courting earlier. As increased GtH levels appear to be concurrent with the onset of courtship behaviour (see following discussion), a sexually stimulated increase in GtH may have occurred before the 2 h blood sample time.

Consistently, more milt could be hand-stripped from male goldfish after 1 to 24 h of contact with a pair of spawning fish than from unstimulated controls. However, elevations of both expressible milt volumes and GtH concentrations did not occur if contact with the spawning pair was prevented, either by placing the male in an adjacent tank where the spawning fish could be only seen (exp. 5.1.4) or separating him from the spawning pair with a perforated partition (exp. 5.1.5). In addition, the GtH and milt responses were similar in males exposed to either a heterosexual or homosexual pair of spawning fish. Taken together, these results suggest that access to the spawning situation must occur to induce these rapid physiological changes. This is different from the situation in male mammals and birds, where exposure to the auditory, visual, or chemical cues of a female are sufficient to elevate LH and T (Harding, 1981; O'Connell, 1981). In other fishes, female pheromones also are sufficient to induce behavioural changes in the male (Liley, 1982), although it is not known whether endocrine stimulation is involved.

The experimental design used for most of study 5.1 does not allow the conclusion that increases in milt and GtH were caused by sexual stimuli *per se*, as no comparable nonsexual stimulus was given to the control groups. Nonspecific stress can elevate the levels of several hormones in mammals, including LH (Krulich et al., 1974; Euker et al., 1975; Turpen et al., 1976). Presumably, any stress effects due to handling

would be equal among treatments in the present experiments; but males placed in contact with a spawning pair additionally may have experienced some nonsexual, socially-induced stress. However, two observations suggest that the increases in GtH and milt were specifically related to sexual behaviour. Instead of exposing males to a spawning pair, exp. 5.1.1 presented experimental fish with either another male or a receptive female, stimuli that presumably are equal socially but unequal sexually; a GtH response was seen only in the female-exposed males. Furthermore, males that were sexually inactive when exposed to a spawning pair were not different from isolated fish in their GtH and milt levels (Fig. 5.1.6). In contrast, sexually active individuals had GtH and milt levels higher than the isolates or inactive fish, suggesting that sexual activity and elevations in milt and GtH are concurrent events which may share a common activational mechanism. As well, it appears that actual spawning need not occur in order to observe the GtH response. The fish in exp. 5.1.1 were courting but had not spawned by the 20 min sample time, when GtH levels were significantly elevated.

Similar neural mechanisms may be involved in mediating rapid rises in GtH evoked by external stimuli in both male and female goldfish. In females, GtH levels rose rapidly prior to ovulation (Stacey et al., 1979a), and the number of fish ovulating spontaneously was increased by exposure to artificial aquatic vegetation for as little as one light phase (Stacey et al., 1979b). It has been proposed that the ovulatory surge of GtH is controlled both by release from the influence of an inhibitory factor (GRIF) originating in the preoptic area and by stimulation by a hypothalamic releasing factor (GRF) (Peter and Paulencu, 1980). As courtship activity and the GtH increase occur together in male goldfish, modulation of GRIF and/or GRF action may be accomplished by the same neural substrates that are activated during male sexual behaviour. Male sexual behaviour in goldfish depends upon the integrity of an area in the supracommissural telencephalon (Vs-pVv) (Chapts. II and III). While lesion of the Vs-pVv does not alter resting levels of GtH (Chapt. III), this area may mediate the phasic GtH rise induced in males by courtship behaviour and, possibly, in females by aquatic vegetation. In mammals, the amygdala appears to mediate the facilitatory effects of mating stimuli on ovulation (Raisman and Field, 1972); parallels between the behavioural function of the amygdala and the Vs-pVv of goldfish have been noted (Chapt. III).

As sexually-induced elevations in milt and GtH were concurrent (with the possible exception of exp. 5.1.4) and as replacement therapy with gonadotropin reinstates spermiation in hypophysectomized goldfish (Yamazaki and Donaldson, 1968; Billard, 1977; Billard et al., 1982), the sexually-stimulated release of GtH may be responsible for the increased volume of expressible milt. The present results on the time course of milt volume increases following gonadotropin injections were equivocal regarding this possibility. Male goldfish injected with either hCG or cGtH did not show a significant increase in milt volume until between 3 and 6 h postinjection (Fig. 5.2.1), suggesting that the action of endogenous GtH would be too slow to account for the elevation in milt volume seen after 1 h of sexual stimulation (Fig. 5.1.2). Moreover, the dose of GtH used was sufficient to cause a serum level of about 35 ng/ml 1 h after i.p. injection into mature male goldfish at 12°C (Cook and Peter, 1980a), a concentration at least twice as high as the endogenous levels found in 4/5 of the present experiments. However, the results from a subsequent experiment first detected increased milt volumes between 1 and 3 h after injection of cGtH or pituitary extract, which, allowing for a delay due to uptake of hormone from the peritoneum, is within the time range seen for the sexually stimulated milt response. It is not clear why the same dose of cGtH at the same temperature yielded two different patterns of milt response. The males in the latter experiment had higher initial volumes of milt, suggesting that they were more mature and possibly more responsive to exogenous gonadotropin administration.

Two unexpected results were found in the injection experiments. First, over the first 12 h after injection, similar volumes of milt were collected from fish injected with 25 ng/g cGtH and goldfish pituitary homogenate, even though the cGtH-equivalent dose of the latter would have been about 250 times greater than the cGtH injection, as estimated from the average pituitary GtH content (Cook and Peter, 1980b). At 24 h, however, the milt volume of the pituitary extract-injected group had exceeded that of cGtH-injected fish, suggesting that uptake of hormone from a crude homogenate is slower than from a relatively pure saline-diluted preparation or that the response was at a maximum over the first 12 h and differences only appeared when the effect of cGtH began to wane. Second, gonadotropin or pituitary homogenate treatment did not increase the seminal plasma content, as described for the carp (*Cyprinus carpio*)

(Clemens and Grant, 1965). Possibly the high variability within each treatment and the difficulty in accurately measuring the small volumes of milt collected from the saline-injected animals masked any changes in % seminal plasma due to hormone treatments. On the other hand, the fish in this study were already reproductively mature, and presumably the sperm ready for release had already undergone optimal dilution. Thus, hormone administration to fish in this state may have accelerated the process of milt production without altering the fluid content of the product.

When pituitary extracts or gonadotropin (salmon GtH or hCG) were injected into male carp or goldfish, the water content increased uniformly along the length of the testes, the epithelial cells of the sperm ducts hypertrophied, spermatozoa detached from the duct walls, presumably due to fluid secretion by the duct cells, and the volume of expressible milt, milt plasma, and water content all increased (Clemens and Grant, 1964; 1965; Yamazaki and Donaldson, 1968; Billard, 1976; 1977). Although not all of the above parameters have been measured in the same study and few measurements have been taken at times less than 24 h postinjection, these events are thought to occur concurrently during spermiation, (for example, milt volumes would not be elevated hours before testicular hydration). Thus, if sexually stimulated milt volume increases are mediated by GtH, then these increases should be accompanied by increases in testicular water. Study 5.3 found that the increased milt volumes seen 4 h after the onset of sexual stimulation were not paralleled by an increase in testicular water. It is possible that testicular hydration occurred but was not detected in these sexually stimulated fish, as the technique used may be restricted to the measurement of relatively large changes in testicular water, such as those seen 12 h after hCG treatment. Alternatively, whereas testicular hydration and increased milt production may be a mechanism by which sexual stimuli maintain elevated volumes of expressible milt, the initiation of these elevations may occur via a GtH-independent mechansim, such as increased movement of milt into the posterior portion of the sperm duct for storage.

Accumulation of milt in readiness for release is probably accomplished by contractions of the sperm duct (Billard et al., 1982). In fish, hormones of the neurohypophysis, urophysis, and the sympathetic nervous system have been shown to influence sperm duct contractility and sperm "release" as a result of hand-stripping

(Berlind, 1972; Billard, 1977), and thus may be potential mediators of sexually stimulated milt volume increases. As previously discussed, the results of injecting crude pituitary extract into goldfish were equivocal; however, the lack of a significant increase in milt volumes 1 h after injection of supraphysiological doses of hormone casts doubt upon the importance of hormonal mediation in the fast sperm response. The injection of urophyseal homogenate or adrenalin had no effect.

Demski and coworkers have evoked sperm release in sunfish (*Lepomis*) and goldfish by electrically stimulating a tract running from the preoptic area through the brain stem to the rostral spinal cord (Demski et al., 1975; Demski, 1978; Demski and Hornby, 1982). Removal of the pituitary and urophysis or paralysis with *d*-tubocurarine had no effect on the evoked sperm release, and preliminary results indicated that the response may be mediated by a cholinergic sympathetic system (Demski and Hornby, 1982). Perhaps a similar pathway mediates loading of the posterior sperm ducts and, consequently, a rapid increase in expressible milt volume during sexual stimulation.

In summary, the performance of sexual behaviour is correlated with the rapid elevation of GtH concentrations and expressible milt volumes, which may act as a positive feedback system to maintain sexual readiness. Elevated GRF, GtH, or sex steroid levels may facilitate reproductive functions that are rate-limited by hormone availability. While steroid hormones induce sexual behaviour in regressed male goldfish (Chapt. IV), the males in the present studies obviously had sufficient levels of reproductive hormones to allow the performance of sexual behaviour. However, perhaps subsequent sexual activity can be facilitated by stimulation of the hypothalamo-pituitary-gonadal axis, suggesting that a physiological mechanism might participate in mediating the facilitatory effects of experience. The performance of sexual behaviour also increases the amount of milt stored in readiness for spawning, perhaps both by moving milt into the posterior portion of the sperm duct and by increasing milt production via the elevation of GtH.

VI. GENERAL DISCUSSION

The foregoing chapters have presented new information regarding the neural and hormonal control of spawning behaviour in the male goldfish (*Carassius auratus*) and the effects of spawning performance on reproductive condition. This chapter summarizes that information and gives a general interpretation of the results.

A number of studies (reviewed in Chapt. II) have implicated the nuclei of the ventral telencephalon and preoptic area in the control of male spawning behaviour in fish; but when lesions were placed stereotaxically in these nuclei of male goldfish, only lesions in the *area ventralis telencephali pars supracommissuralis* (Vs) and *area ventralis telencephali pars ventralis* (Vv) significantly reduced the proportion of males spawning. Furthermore, a probability analysis (Fig. 2.2, which I believe is a unique approach, cf. Wolf and Gollob, 1980) showed that the effective area, the Vs and posterior Vv (Vs-pVv), corresponded precisely with the only site in the goldfish telencephalon that concentrates sex steroids (Kim et al., 1978a), thus providing the first direct evidence for a reproductive role for this area (Chapt. II).

Lesions of the Vs-pVv did not destroy the motor capability for spawning because about two-thirds of Vs-pVv lesioned males showed the complete spawning sequence on at least one of the weekly spawning tests (Chapt. II). Neither was the reproductive endocrinology of these animals appreciably altered by these lesions, as the GSIs and serum GtH concentrations were similar in lesioned and sham lesioned goldfish (Chapt. III). The following circumstantial evidence suggested that Vs-pVv lesions impaired male sexual behaviour by disrupting the processing of critical olfactory cues. In goldfish, the lateral portions of the Vs-pVv lie adjacent to one terminal field of the medial olfactory tracts (Oka et al., 1982) and detailed pictures of olfactory tract projections in the paradise fish (*Macropodus opercularis*) implied that the steroid-concentrating cells in the Vv could receive olfactory information (Davis et al., 1981). Furthermore, section of the medial, but not the lateral, olfactory tracts (N.E. Stacey and A.L. Kyle, unpublished results), as well as Vs-pVv lesions, produced deficiencies in male spawning behaviour.

Although Vs-pVv lesions did not impair the perception of a food odor (Chapt. III), the question of whether lesioned males can smell sexual odors is still unanswered. In this

regard, it is interesting to note that the ability to perceive a sexual odor may vary with the hormonal state. Based on the preference of goldfish for the odor-containing arm of a Y maze, Partridge et al. (1976) determined that mature, spermiating males responded to odors from both food and an ovulated female, while nonspermiating males responded only to the food odor. As well, the nonspermiating fish apparently were less sensitive to odors in general. This correlates with electrophysiological recordings from the olfactory bulb of goldfish, which showed that the amplitude of the bulbar electroencephalogram (OB-EEG) was proportional to the sexual maturity of the male (Goff, 1979). This is likely an effect of increased steroid levels, as steroid treatment altered the OB-EEG seen in response to lavage of the nares with a saline solution (Oshima and Gorbman, 1966; Hara, 1967). Furthermore, the sex steroid-concentrating cells of the ventral telencephalon may mediate the effect of steroids on olfactory function, as this area sends centrifugal fibres to the olfactory bulbs via the medial olfactory tracts (Oka, 1980) and section of the medial, but not the lateral, olfactory tracts altered the OB-EEG (Hara and Gorbman, 1967).

While impairment of male sexual behaviour by disruption of pheromone perception is a plausible hypothesis for the mode of action of Vs-pVv lesions, the experiments of Chapt. III showed that olfactory-independent behaviours also were affected. Male goldfish that failed to show male behaviour also failed to show female spawning activity; the latter can be evoked in intact males by PG-treatment and is not reduced by section of the olfactory tracts (N.E. Stacey and A.L. Kyle, unpublished results). Moreover, the hyperactivity shown by isolated, intact males was not seen in lesioned animals, suggesting that Vs-pVv lesions disrupted the processing not only of olfactory stimuli necessary for male behaviour, but also of stimuli that regulate other sexual and social activities. All behaviour was not indiscriminantly reduced, however, as feeding in response to a food odor, a non-social activity, was unaffected by Vs-pVv lesions.

Because the Vs-pVv both binds steroid hormones and is involved in spawning behaviour, this site may mediate the potentiating effects of gonadal androgens on sexual behaviour. If implants of androgens into the Vs-pVv could reinstate behaviour in sexually inactive males, then this hypothesis would be supported. However, experiments

designed to test this idea (Chapt. IV) were thwarted by the difficulty in obtaining sexually inactive male goldfish, as castrated males readily regenerated testicular tissue and regained sexual activity, regressed winter fish showed unacceptable amounts of courtship behaviour, and fish kept for extended lengths of time under conditions that promoted extreme sexual regresssion also may have been rendered insensitive to androgen treatment. Nevertheless, the hypothesis of a central site for androgen action was supported by the observation that when pellets were placed in the Vs-pVv, more testosterone-implanted than blank-implanted males spawned, although the difference was not significant (p=0.12). In addition, central implants of the antisteroid drug, enclomiphene citrate, significantly reduced the number of mature males spawning.

Depending on the behaviour and the species involved, conversion of testosterone to either dihydrotestosterone or estradiol is necessary for the stimulation of androgen-dependent behaviour in mammals and birds (Adkins-Regan, 1981). Testosterone also may be acting as a "prohormone" in goldfish, as systemic injection of 11-ketotestosterone, a prominent androgen in fish, appeared to be more effective than either testosterone or estradiol in inducing male spawning. Although enclomiphene citrate is usually considered to be antiestrogenic in mammals (Murad and Haynes, 1980), the possibility that it may compete with the receptors for other steroids in fish remains open. The problem of the hormonal specificity of male spawning behaviour needs further investigation.

Chapt. V showed that male goldfish exposed to sexual stimuli, in the form of a receptive female or a stimulus pair of spawning goldfish, had increased "Con A II" or "maturational" gonadotropin (GtH) levels and expressible milt volumes within 1 h, as compared to nonstimulated males, and the stimulatory effects on GtH and milt persisted for at least 2 and 24 h, respectively. Although similar, transient elevations in gonadotropin levels as a result of sexual stimuli have been observed in birds and mammals (Harding, 1981), this is the first report for fish. The sight, sound, or smell of a female is sufficient to cause an increase in gonadotropin in male birds and mammals, but in goldfish, no elevations of GtH or milt levels were seen if the males were sexually inactive or separated from the spawning pair by either a solid or perforated transparent partition. Furthermore, the presence of a female was unnecessary, as exposure to a

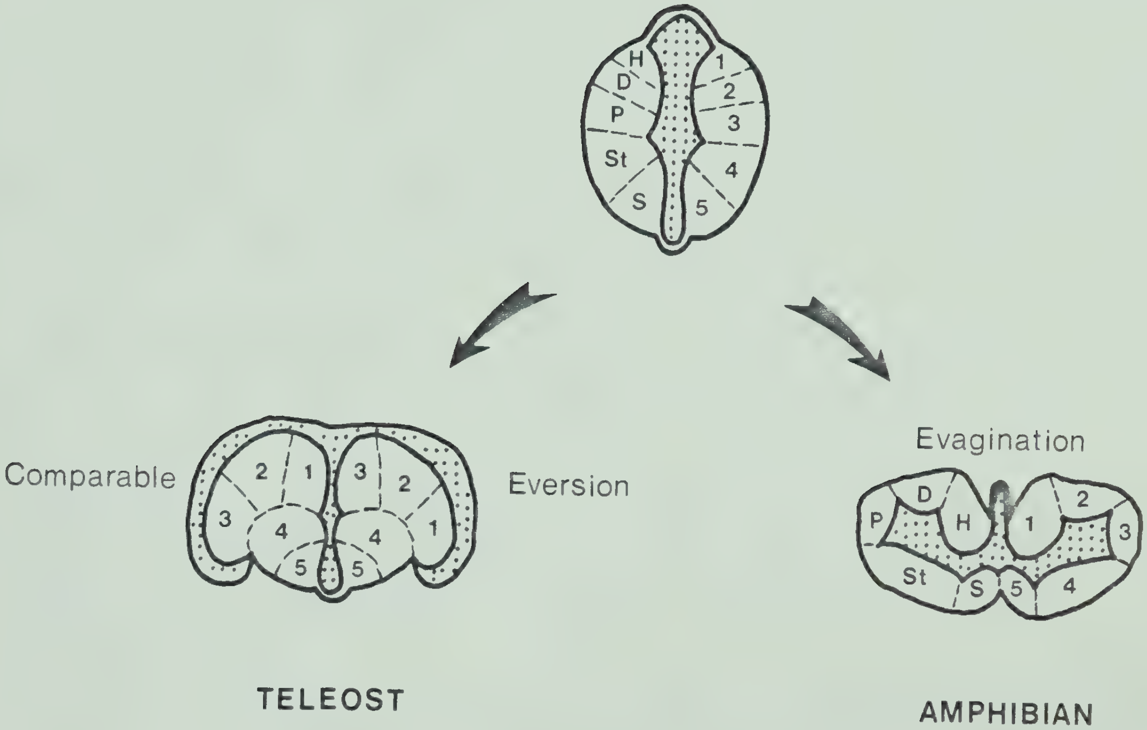
homosexual spawning pair (where a PG-treated male played the female role) was as effective as exposure to a heterosexual pair in elevating GtH and milt levels. Taken together, the results suggest that exposure to the spawning situation, rather than cues specific to a female, caused these physiological changes.

The stimulatory effects of gonadotropin on spermiation are well known (Billard et al., 1981), which suggests that the sexually stimulated increases in expressible milt may be due to the concomitant rise in GtH. Preliminary experiments did not allow any firm conclusions to be made regarding this idea, but the fact that sperm release is a neural event involving the sympathetic nervous system (Demski and Hornby, 1981) suggests that an increase in expressible milt volumes may be the result of both a neurally mediated movement of milt from the anterior portions of the testes into the posterior sperm duct and a hormonally mediated increase in milt production. In addition to facilitating milt production, elevated GtH levels also may potentiate other reproductive functions that are rate-limited by hormone availability.

The unorthodox development of the teleost telencephalon has resulted in diverse interpretations of the anatomical relationships between teleost fish and land vertebrates. Whereas the rostral zone of the primitive neural tube of most vertebrates evaginates bilaterally to produce paired ventricles, in teleosts it everts to yield a T-shaped ventricle that is enclosed dorsally by the tela choroidea (Fig. 6.1). The "comparable" hypothesis suggests that the topology of the major forebrain primordia in teleosts is directly homologous to other vertebrates, thus the hippocampus always would be situated dorsal to the septum, even though this juxtaposition of neural fields in other vertebrates has occurred because of the evagination process. In contrast, the "eversion" hypothesis maintains that the forebrain primordia retain their relative positions throughout the eversion process, resulting in a medio-lateral reversal of the dorsal structures relative to land vertebrates (Fig. 6.1). Most embryological, anatomical, and histochemical studies support the latter interpretation (Northcutt, 1981).

Even though the neural fields of the ventral telencephalon would be subjected to less rearrangement as a result of the eversion process, there are still discrepancies in the literature regarding their homologies with other vertebrates. As Campbell and Hodos (1970) have emphasized, a number of criteria should be used to establish homology

Fig. 6.1. Comparison of the way in which the topology of the telencephalon might be preserved during development of a teleost and an amphibian. The amphibian brain shows evagination and inversion; the teleost brain shows two extreme views of topographic organization. D – dorsal pallium or neocortex, H – medial pallium or hippocampus, P – lateral pallium or pyriform cortex, S – septal region, St – striatum, 1–5 – corresponding major telencephalic subdivisions. (Adapted from Schroeder, 1980 and Northcutt, 1981)



between two structures, such as similarity of fibre connections, topology, embryology, histochemistry, and electrophysiology, and behaviour. The results of this thesis can offer information regarding the last criterion.

As summarized in Chapts. II and III, the Vs–pVv of goldfish and the corticomedial amygdala of mammals appear to have similar functions in the control of male behaviour; both sites receive olfactory input and male sexual behaviour is blocked either by removal of this input or by lesions in the amygdala or the Vs–pVv. As well, both sites have similar efferent connections, although the pattern in goldfish has not been described in detail, and both bind sex steroid hormones (Kim et al., 1978a – goldfish; Pfaff and Keiner, 1972 – mammals). Nonolfactory behaviours, such as female spawning and swimming activity during isolation, also are reduced by Vs–pVv lesions in the goldfish. These observations are consistent with theories of amygdaloid function. In a discussion of the functional significance of the amygdala in nonmammalian vertebrates, Gloor (1971) suggests that:

> Probably, for the majority of mammals below the level of primates, no sensory system has such a profound impact on behavior as olfaction. It provides the cues not only for the searching and ingestion of food, but also for reproductive behavior, maternal behavior, avoidance behavior and, most importantly, also for the integration of the individual within the social group. Even in fish, olfaction ..... provides the neural basis for plasticity of behavior.....[such as] individual recognition, cooperative behavior and dominance (p. 432)

Gloor hypothesized that since many motivational mechanisms are activated by the olfactory sense, the structures involved in olfactory processing gradually took on the general function of assessing sensory signals and activating pathways at lower brain levels that organize motor output. Thus, a wide variety of activities may be disrupted following amygdaloid lesions, but there is no obvious difficulty in the performance of a particular response when it does occur (Cormier, 1981), an observation also noted for the behaviour of Vs–pVv lesioned goldfish. These types of observations led Cormier to suggest that the primary role of the amygdala is one of stimulus processing, particularly of cues involved in species–specific behaviour.

Whether or not behavioural parallels suggest homology between part or all of the Vs-pVv in goldfish and the amygdala of mammals, analogies drawn between these two structures provide a framework to assist the construction of meaningful hypotheses. For example, in view of the relationship of the Vs-pVv with spawning behaviour and the latter with rapid elevations of GtH levels, the facilitatory effect of the amygdala on gonadotropin secretion in mammals (Komisaruk et al., 1981) suggests that the Vs-pVv also may play a role in regulating the GtH secretion of goldfish.

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